NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR
Annual Technical Report
1995

Submitted by

Nicolas G. Bazan, M.D., Ph.D.
Project Director

Period Covered: 20 September, 1994, through 19 September, 1995

Cooperative Agreement DAMD17-93-V-3013

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United States Army Medical Research and Development Command
(Walter Reed Army Institute of Research)

and

Louisiana State University Medical Center
Neuroscience Center of Excellence

Volume 3 of 8

The Neuro-immunology of Stress, Injury and Infection

Project Directors:
Bryan Gebhardt, Ph.D.
Daniel J.J. Carr, Ph.D.

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TABLE OF CONTENTS

Page Nos.

ANIMAL USE
20 SEPTEMBER, 1994, THROUGH JULY, 1995

DAMD17-93-V-3013

The experimental animals used during this period for the project, Neural Responses to Injury: Prevention, Protection, and Repair, **Subproject: Neuroimmunology of Stress, Injury and Infection**, are as follows:

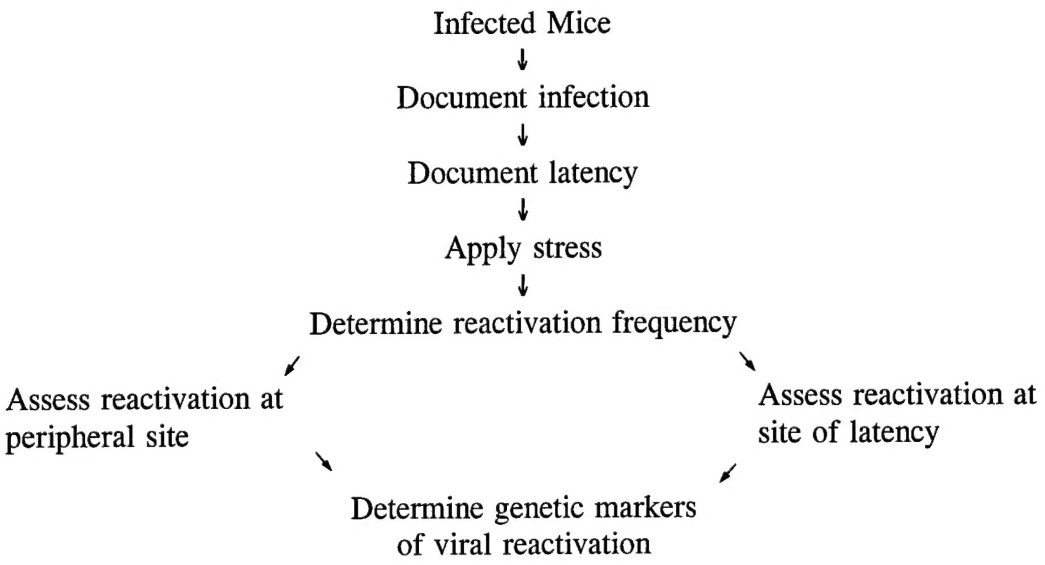
Species	Number Allowed	Number Used	LSU IACUC #
Mouse	270	270	1019

Bryan W. Jakeman
Investigator Signature

ABSTRACT

The hypothesis on which this investigation is based is that stressors such as transient temperature changes and restraint signal the central nervous system eliciting the release of catecholamines and adrenal steroids which, in turn, affect the immune system resulting in the reactivation of latent viruses. Employing a mouse model of stress-induced reactivation of herpes simplex virus type 1 (HSV-1), we are determining the time course of viral reactivation relative to the alteration of immune parameters including lymphocyte functions and numbers. Specifically, we are correlating the expression of various immunomodulatory cytokine genes with the levels of neuroendocrine monoamines, as well as the activation of the hypothalamic-pituitary-adrenal (HPA) axis and relating these to the reactivation of infectious virus in the nervous system. Alterations in serum corticosterone and shifts in monoamines in the brains, trigeminal ganglia, and brain stems of latently infected and reactivated mice following the application of stress are being studied. Differences between control (not stressed) and stressed animals are being determined relative to the incidence of viral reactivation and the affect of stress on immunological regulation of the reactivation process. The knowledge gained from this investigation will provide an understanding of the interaction between the nervous system, the neuroendocrine system, and the immune system during times of stress at the molecular and cellular levels.

FIRST YEAR FLOW CHART



First Year Goal: To determine the effects of a brief period of thermal stress (10 minutes at 43°C) and restraint stress (60 minutes) as indirect mediators of HSV-1 reactivation from neural tissues.

These experiments were designed to allow us to determine the frequency of viral reactivation following moderately stressful events and to establish a baseline from which we can launch a full-scale assault on the analysis of the neuroendocrine-immunologic interactions which take place during stress-induced viral reactivation. Groups of 10 mice in each group, which had been infected with the McKrae strain of HSV-1 by the ocular route 35 days previously were subjected to one of the stress protocols. At 24 hours after the application of the stressor (43°C for 10 minutes or 60 minutes of restraint stress) the groups of mice were sacrificed, the ocular surface swabbed, the globes removed, and the trigeminal ganglia dissected free. Control groups of animals included uninfected, stressed animals, infected animals that were not stressed, and animals which were neither infected nor stressed.

Table 1 below indicates the results of the assays for infectious virus from the

experimental and control groups of animals. For the convenience of the presentation of the data, the results obtained with animals subjected to the two different stress paradigms are separated into Tables 1 and 2. It can be seen that the heat stress paradigm induced a higher percentage of reactivations (80%) as compared to the restraint stress model (40%). This experiment was repeated three times in order to confirm the results and to permit detailed statistical analyses. By analysis of variance (ANOVA) the difference between the reactivation of infectious virus in the tears, ocular tissue, and trigeminal ganglia of infected, stressed animals was significantly different from any of the control groups ($P < 0.005$). Thus, the stressors chosen for use in these investigations are bonafide methods of inducing viral reactivation.

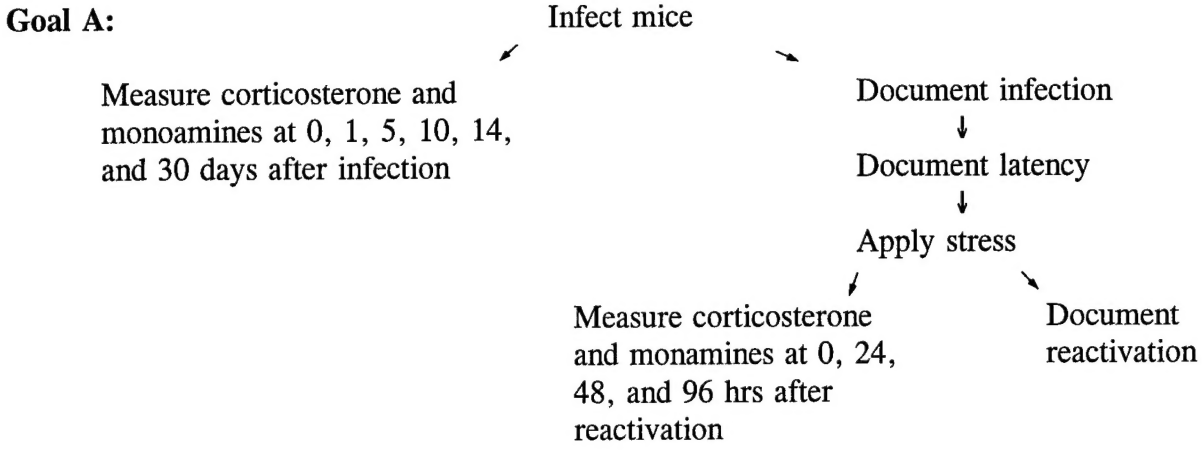
TABLE 1: Viral Reactivation Following Heat Stress

Treatment Groups	Presence of Infectious Virus In:		
	Tear Film	Corneas	Trigeminal Ganglia
Infected, stressed	4/10	6/10	8/10
Infected, not stressed	0/10	0/10	0/10
Not infected, stressed	0/10	0/10	0/10
Not infected, not stressed	0/10	0/10	0/10

TABLE 2: Viral Reactivation Following Restraint Stress			
Treatment Groups	Presence of Infectious Virus In:		
	Tear Film	Corneas	Trigeminal Ganglia
Infected, stressed	1/10	3/10	4/10
Infected, not stressed	0/10	0/10	0/10
Not infected, stressed	0/10	0/10	0/10
Not infected, not stressed	0/10	0/10	0/10

As a direct consequence of these studies comparing the efficiency of heat and restraint stress on viral reactivation, several manuscripts have been prepared. Most importantly it has been documented that herpes virus reactivation can be accomplished with a frequency which is adequate for investigation in this overall project. We have conducted a series of studies testing various antiviral drugs and, in a particularly revealing investigation, have shown that ß-adrenergic receptor blocker, propranolol, can suppress viral reactivation following heat stress treatment of latent mice. These investigations are being continued to further define the physiologic and molecular mechanisms of viral reactivation.

SECOND YEAR FLOW CHART



Second Year Goal: A. To determine the neuroendocrine mechanism of stress-induced viral reactivation by measuring corticosterone and monoamine levels in the serum and nervous tissues of latently infected, stressed animals.

The overall goal of the experiments conducted as part of this specific aim are to determine the types of neuromediators which are involved in viral reactivation and also which are involved in modulating the immune response so as to permit viral reactivation. To determine the possible interactions between latent HSV-1 infection and the stress response of mice, the induction of endocrine and paracrine mediators was compared in four treatment groups: infected, stressed mice; infected, not stressed mice; uninfected, stressed mice; and uninfected, not stressed mice. Using the heat stress paradigm described above, it was found that 24 hours after application of the stressor that serum corticosterone in the infected mice were significantly higher as compared to uninfected mice ($P < 0.05$). The data from a typical experiment in this series is shown in Table 3 below.

TABLE 3: Effect of Heat Stress-Induced Viral Reactivation on Corticosterone Levels*			
	HSV-1 Infected	Not Infected	Averages
Stressed	102.3	38.5	70.4
Not Stressed	63.1	52.2	57.7
Averages	82.7	45.3	

Two-way ANOVA:

H_0:	Probability:
X_{av} (Stressed) = X_{av} (Not Stressed)	$p = 0.28$
X_{av} (Infected) = X_{av} (Not Infected)	$p = 0.015$

*nanograms/ml

It can be seen that there was a statistically significant elevation in corticosterone levels in the infected mice. Additional studies of the monoamine levels in the brain stems of the experimental and control groups of animals have been conducted 24 hrs after application of the stressor. Catecholamine levels were not found to be significantly altered in any of the treatment and control groups investigated to date (Table 4).

TABLE 4: Effect of Heat Stress-Induced Viral Reactivation on Brainstem Catecholamine Levels*				
Treatment Groups	NE‡	SHIAA	DA	5HT
Infected, stressed	931	228	31.3	220
Infected, not stressed	1030	244	88.3	280
Not infected, stressed	742	131	23.2	186
Not infected, not stressed	966	227	56.6	251
One-way ANOVA: $X_a=X_0=X_0=X_a$	$p = 0.77$	$p = 0.58$	$p = 0.17$	$p = 0.69$

‡NE = norepinephrine; 5HIAA = 5-hydroxyindoacetic;
DA = dopamine; 5HT = 5-hydroxytrytophane

*picograms/milligram

Further studies of the catecholamine and corticosterone levels at earlier time points and the significance of this response to viral reactivation are in progress.

Our initial investigation into the modulation of the immune response mediated by neuroendocrine mediators as a response to heat stress has produced some meaningful results. Among other things, we have investigated the production of interleukin 6 (IL-6) at the cellular and molecular level following application of stress to latently infected mice. We have noted that serum concentrations of corticosterone and IL-6 in uninfected, stressed mice were positively correlated ($r = 0.87$), but were negatively correlated ($r = -0.28$) in infected, stressed mice. These data are shown in Table 5 below. This observation suggests that an underlying difference exists between the regulation of IL-6 and corticosterone when one compares latently infected and uninfected animals following exposure to the stressor.

TABLE 5: Correlation Coefficients of Serum Corticosterone and IL-6 Levels in Heat Stressed, Infected Animals

Treatment Groups	r Values
Infected, stressed	$r = -0.28$
Infected, not stressed	$r = -0.57$
Not infected, stressed	$r = 0.87$
Not infected, not stressed	$r = 0.27$
T-test: H_0: $r_{(\text{infected/stressed})} = r_{(\text{not infected/stressed})}$	

Molecular biological analysis of the expression of the IL-6 gene in the trigeminal ganglion of stressed latently infected mice compared to stressed, uninfected mice, as well as mice that were not stressed but latently infected, reveal that there is an apparent reduction in

the expression of the IL-6 message in the latently infected, stressed animals (Fig. 1). Since,

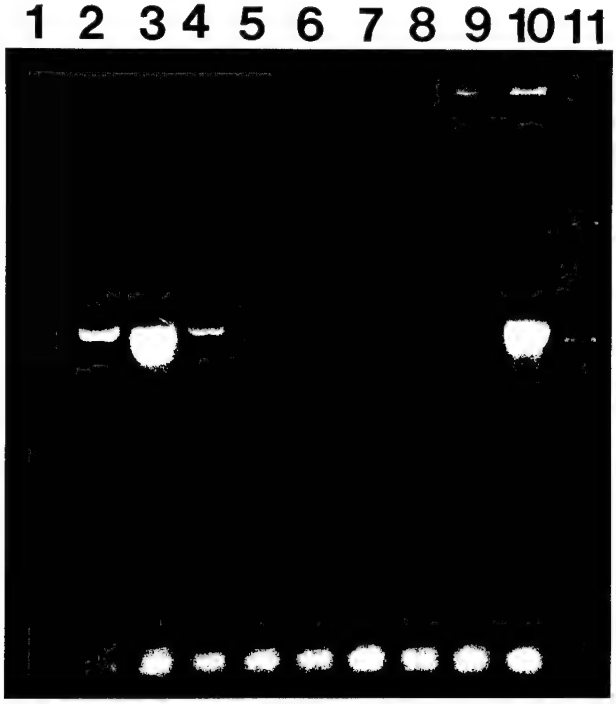
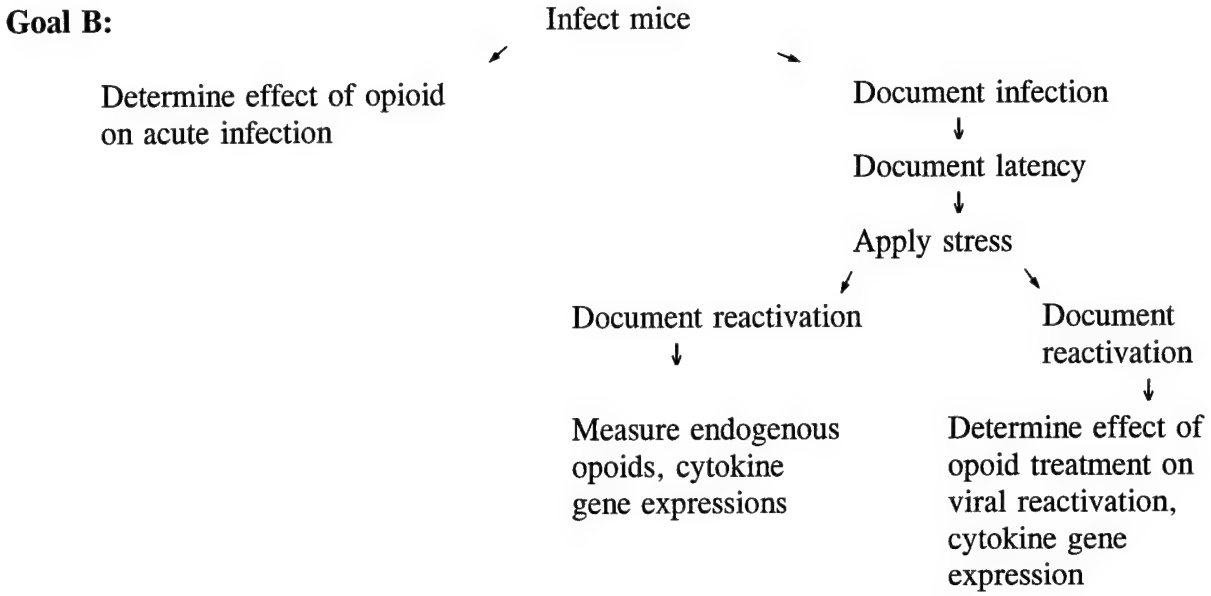


Figure 1. Reverse transcription-polymerase chain reaction (RT-PCR) for IL-6 mRNA in trigeminal ganglia. Lane 1 = Molecular weight markers; Lane 2 = Not infected, not stressed; Lane 3 = Not infected, stressed; Lanes 4,5 = Infected, not stressed; Lanes 6-9 = Infected, stressed; Lane 10 = Positive control; Lane 11 = Molecular weight markers.

the trigeminal ganglion is one of the sites of latency of HSV-1, we speculate that perhaps the virus can in some way alter the host's production of immunomodulatory mediators including the pro-inflammatory cytokine IL-6 and, by extension, corticosterone. Currently, competitive PCR is being carried out to quantitate differences between the levels of IL-6 expression in the trigeminal ganglion of the various treatment groups. We can now detect as few as 100 copies of template. Further studies of the role of cytokines (TNF-α, IL-1, and IL-6) and neuroendocrine mediators in viral reactivation during the host immune response are in progress.

SECOND YEAR FLOW CHART



Our investigations of the role of cytokine gene activation following heat stress have yielded a number of interesting results. A manuscript is in preparation detailing the time course of cytokine gene expression in the cornea and trigeminal ganglion of mice during acute, latent, and reactivated viral infection in mice. In essence, we have found that several of the proinflammatory cytokines are expressed in the cornea and trigeminal ganglion during acute viral infection and that subsequently interleukin 2, interleukin 10, and gamma interferon are produced once the infected host's immune system mobilizes lymphocytes which migrate into the sites of infection. Upon viral reactivation, we find that there is only a very transient expression of interleukin 6 and the chemokine RANTES, followed by a spike of gamma interferon production. Only if the viral reactivation is prolonged by manipulation of the host's immune response do we find anything more than a very brief burst of cytokine gene activity in the trigeminal ganglion following reactivation.

Second Year Goal: B. To investigate the role of the neuroendocrine system following morphine exposure.

The approach that we have taken to investigate the role of the neuroendocrine system in viral latency and reactivation has been through the use of hypothysectomized and adrenalectomized animals. In a study which is now being prepared for publication, we have found that adrenalectomy significantly impairs the development of antiviral immunity, both cellular and humoral, and as a result infectious virus is present in at both the ocular surface and in the trigeminal ganglion for longer periods of time. Hypothysectomized animals appear to respond relatively normally with the development of an antiviral immune response and further studies on the compensatory mechanisms involved are in progress. Studies of viral reactivation following application of the stress protocol are being pursued in such animals.

As a direct result of the Department of Defense support for our study, we have been able to provide new insights into the role of morphine-mediated suppression of cellular immunity in mice. These studies have culminated in the submission of four abstracts, the presentation or planned presentation of our findings at three national meetings, and the submission of four manuscripts. In addition, pertinent studies are near completion in the cloning of an orphan opioid receptor from lymphocytes which may lead to insights as to the role of opioids (exogenous and endogenous) on the regulation of immunocompetence either constitutively or following bacterial, parasitic, or viral infection. A summary of the findings to date are presented below.

Opioids, such as morphine, are a known chemical stressor that has a detrimental effect on the immune system. In fact, early research by Bryant, Bernton, and Holaday (1987; 1991) of the Division of Neuropsychiatry of Walter Reed Army Institute showed that morphine was a potent immunosuppressive drug which when administered *in vivo* suppressed mitogen-induced lymphocyte proliferation and delayed-type hypersensitivity reactions through

the activation of the HPA axis. To further these initial observations, the P.I. (Gebhardt) and co-P.I. (Carr) began a series of studies to investigate the potential role of the sympathetic nervous system and HPA axis involvement in morphine-induced suppression of cell-mediated immunity concentration on natural killer (NK) activity and the generation of cytotoxic T lymphocytes (CTLs). Both of these effector populations are involved in viral and tumor surveillance making them key figures in reducing the potential incapacitation as a result of viral reactivation or infection. The results of these studies showed that morphine suppressed NK activity through central activation of α-adrenoreceptors (Carr et. al, 1994). Additional studies were conducted on chronic morphine exposure and CTL activation using an allogeneic mouse model. The results of these studies show that chronic morphine exposure significantly suppresses CTL activity following alloimmunization in mice. The suppression is, in part, due to a reduction in intracellular signalling following effector - target cell conjugation as well as the release and synthesis of enzymes associated with the "lethal hit". Using a pharmacological approach, the suppression can be antagonized using the μ-opioid receptor antagonist, β-funaltrexamine, but not the δ-opioid receptor antagonist, BNTX. Likewise, serum measurements of corticosterone and DHEA from the vehicle- and morphine-treated mice suggested aberrant adrenal function in the morphine-treated animals. The biological significance of these results is substantiated by the study showing that mice infected with HSV-1 and exposed to morphine succumb sooner and with increased frequency, as compared to vehicle-treated, infected mice. These results have helped establish potential mechanisms at a systemic, cellular, and molecular level that may be altered following chronic morphine exposure.

The observations showing that morphine-induced suppression of immunocompetence is mediated through central (brain) pathways has led us to investigate the relevance of the

hypothesized presence of the opioid receptor(s) on cells of the immune system. Recently, the P.I. and co-P.I. generated oligonucleotide primers specific for transmembrane 3 and 5 and the δ-opioid receptor and used these oligonucleotides to detect potential transcripts in stimulated and unstimulated lymphocytes. By RT-PCR, a product of 381 pb was generated. Subsequent primers were made and used to obtain a full-length cDNA which has >90% sequence homology to an orphan opioid receptor cloned from mouse brain. Northern gel analysis of RNA obtained from splenic lymphocytes indicates the transcripts are present but in reduced numbers relative to brain extracts (Halford, Gebhardt, & Carr, manuscript in preparation). These results will be instrumental in establishing a direct link between the endogenous lymphocyte/macrophage-derived proopiomelanocortin hormones, the endorphins, and the potential autocrine- or paracrine-oriented feedback on cells of the immune system as well as the potential direct interaction between exogenous opioids (e.g., morphine) and cells of the immune system.

Currently, a kinetic study of the effects of morphine on CTL activity in alloimmunized mice is underway. In addition, the involvement of corticosterone in this model system is under analysis. The results would suggest that the acute administration of morphine to mice prior to alloimmunization dramatically suppresses peritoneal CTL activity. Serum corticosterone levels are elevated in the morphine administered animals relative to vehicle-treated controls at the 2 h time point but not 12 h or 120 h following morphine administration. To confirm the role of corticosterone involvement in the morphine-mediated suppression, the corticosterone synthesis inhibitor, cyanoketone is currently under study. It is anticipated that these studies will be concluded by October 1, 1994. The results of this investigation will be directly applicable to the studies involving viral reactivation and the neuroendocrine systems involved. Further studies regarding the role of peripheral and

central α-adrenergic pathways in the reactivation of HSV-1 from latency and the effect on the immune system are in progress.

Third Year Goal: To determine the effect of externally applied catecholamines and corticosterone antagonists and agonists in terms of their capacity to prevent or elicit viral reactivation in stressed and nonstressed animals.

THIRD YEAR FLOW CHART

Goal A:

Infect mice
↓
Document infection

Determine effect of exogenous catecholamines and corticosterone agonists, antagonists on acute infection, development of antiviral immunity and cytokine production

Document latency
↓
Apply stress
↓
Determine effect of exogenous catecholamine and corticosterone agonists on viral reactivation, secondary immune response and cytokine production

Goal B:

Infect mice
↓
Document infection
↓
Document latency
↓
Document immunity-
A. Humoral
B. Cellular
↓
Apply stress
↓
Document reactivation
↓
Determine change in antiviral immunity following reactivation: 0, 7, 14, and 21 days

We have made excellent progress in the first two years of this investigation. So much so that, in fact, investigations on the third year goals are beginning at the present time. We are presently testing the levels of cellular and humoral immunity during latency and viral reactivation in pharmacologically normal mice. Shortly, investigations of the affects of exogenous catecholamines and corticosterone agonists and antagonists will begin. We fully expect that these studies will provide considerable insight into the role of catecholamines and corticosterone in the maintenance of latency and in viral reactivation following stress. Since we have already shown that the ß-adrenergic receptor blocker, propranolol, can interfere with heat stress induced viral reactivation, we feel now that we are in an excellent position to determine the physiological and molecular linkages between stress and reactivation of herpes virus. The results of these studies should provide meaningful information regarding the development of new therapeutic paradigms for preventing viral reactivation in human beings.

PUBLICATIONS:

1. Gebhardt BM, Kaufman HE: Propranolol suppresses reactivation of herpesvirus. Antiviral Research. In press.

2. Gebhardt BM, Wright GE, Xu H, Focher F, Spadari S, Kaufman HE: 9-(4-Hydroxybutyl)-N^2-phenylguanine, HBPG, a thymidine kinase inhibitor suppresses herpes virus reactivation in vivo. Antiviral Research, submitted.

3. Gebhardt BM, Kaufman HE, Hill MJ: Effect of acyclovir on thermal stress-induced viral reactivation. In preparation.

4. Gebhardt BM: Neuroendocrine modulation of ocular herpes virus infection. In preparation.

5. Gebhardt BM, Wall RA: Cytokine gene expression in teh cornea and trigeminal ganglia during acute herpes virus infection. In preparation.

6. Carr DJJ, Gebhardt BM, Paul D: *Alpha* adrenergic and *Mu*-2 opioid receptors are involved in morphine-induced suppression of splenocyte natural killer activity. J Pharmacol Exp Therapeut 264(3):1179-1186, 1993.

7. Carr DJJ, Mayo S, Gebhardt BM, Porter J: Central *a*-adrenergic involvement in morphine-mediated suppression of splenic natural killer activity. J Neuroimmunol 53:53-63, 1994.

8. Carpenter GW, Garza Jr HH, Gebhardt BM, Carr DJJ: Chronic morphine treatment suppresses CTL-mediated cytolysis, granulation, and cAMP responses to alloantigen. Brain, Behav, & Immun 8:185-203, 1994.

9. Halford WP, Gebhardt BM, Carr DJJ: Functional role and sequence analysis of a lymphocyte orphan opioid receptor. J Neuroimmunol 59:91-101, 1995.

10. Baker ML, Carr DJJ, Gebhardt BM: Morphine suppresses peritoneal and splenic CTL activity in a dose dependent fashion in alloimmunized mice. PsychoNeuroImmunology Research Society, November 17-20, 1994, Key Biscayne, FL.

11. Gebhardt BM: Molecular and cellular neuroimmunopathology of herpes virus infection. FASEB J 7(3):A494, 1993.

12. Gebhardt BM: Lymphocytes bind to neural cell adhesion molecules on virus-infected neurons. Thirty-third Annual Meeting of the American Society for Cell Biology, December 11-15, 1995, New Orleans, LA, pp. 59.

13. Gebhardt BM, Hill JM: Neuroimmune control of herpes simplex virus reactivation. J Immunol 150(8):Part II:197A, 1993.

14. Gebhardt BM, Wall RA: A direct method for isolation and amplification of mRNA from the cornea and neural ganglia. Invest Ophthalmol Vis Sci Suppl 34(4):1347, 1993.

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16. Zhang X, Gebhardt BM, Bouterie AM, Hill JM: T lymphocytes recognize LAT expressing neurons. Invest Ophthalmol Vis Sci Suppl 34(4):1349, 1993.

17. Wen R, Bouterie AM, Liu WG, Halford WP, Gebhardt BM, Hill JM: Analysis of herpes simplex type 1 (HSV 1) DNA and latency associated transcripts (LAT) in the autonomic ganglia of rabbits harboring latent virus. Invest Ophthalmol Vis Sci Suppl 34(4):1350, 1993.

18. Bouterie AM, Halford WP, Zhang X, Wen R, Gebhardt BM, Hill JM: Increase in LAT expressing neurons during HSV 1 latency. Invest Ophthalmol Vis Sci Suppl 34(4):1378, 1993.

19. Gebhardt BM: Evidence for antigenic cross reactivity between herpes virus and the acetylcholine receptor. FASEB J 8(5):A976, 1994.

20. Gebhardt BM, Hill JM: Antiviral immunity modulates ganglionic reactivation. Invest Ophthalmol Vis Sci Suppl 35(4):1397, 1994.

21. Hill JM, Gebhardt BM: LacZ gene expression during HSV-1 infection of the neural ganglia. Invest Ophthalmol Vis Sci Suppl 35(4):1397, 1994.

22. Gebhardt BM: CD29/B7 co-receptor interaction during the development of immunity to HSV-1. FASEB J 9(3):A529, 1995.

23. Varnell ED, Kaufman HE, Gebhardt BM, Thompson HW: Propranolol suppression of herpetic recurrences in the rabbit. Invest Ophthalmol Vis Sci Suppl 36(4):S40, 1995.

24. Gebhardt BM, Wall RA: The chemokine RANTES is expressed in the corneas and trigeminal ganglia of mice during HSV-1 infection. Invest Ophthalmol Vis Sci Suppl 36(4):S147, 1995.

COOPERATIVE AGREEMENT NO.: DAMD17-93-V-3013

TITLE: NEURAL RESPONSE TO INJURY, PREVENTION, PROTECTION AND REPAIR

CHAPTER: 6B; NEUROIMMUNOLOGY OF STRESS

INVESTIGATOR: DANIEL JJ CARR, PH.D., PRINCIPAL INVESTIGATOR

REPORT DATE: AUGUST 20, 1995

TYPE OF REPORT: ANNUAL REPORT - SEPT. 21, 1994 THROUGH JULY, 1995

TABLE OF CONTENTS

OVERVIEW

This project is organized around the central hypothesis that stressors (temperature and restraint) have detrimental effects on immunocompetence potentially resulting in the reactivation of latent pathogens. Our working model is a latently-infected herpes simplex virus type-1 (HSV-1) mouse. Our central hypothesis is that the hypothalamic pituitary adrenal (HPA) axis and the sympathetic nervous system (primarily adrenergic pathways) are the parties responsible for HSV-1 reactivation. Alterations in serum corticosterone and shifts in monamines in the brains, trimgeminal ganglia, and brain stems are predicted to correlate to the reactivation status of the stressed mice. In addition, assessing the local immune parameters (specifically cytokine production) and relating their levels to viral reactivation following stress are predicted to be helpful in identifying and elucidating a(the) mechanism associated with viral reactivation. We hypothesize that corticosterone (following HPA activation) and local (trigeminal and sacral ganglia) increases in neurotransmitters (catecholamines) following stress modify the local immune profile leading to virus reaction ultimately resulting in viral shedding and pathogenesis. The pathogenesis can manifests as simple mucocutaneous lesions or potentially develop into more severe complications. Consequently, the degree of discomfort could in fact incapacitate personnel in the form of mental anguish and/or physical impairment or discomfort resulting in poor performance. It is anticipated that by identifying the primary mechanism of viral reactivation, pharmacotherapeutic strategies can be applied reducing or eliminating viral reactivation.

BUDGET OF THIS YEAR'S REPORT

SALARIES:	22,080
TRAVEL:	1,500
OPERATING SERVICES:	3,607
SUPPLIES:	10,908
EQUIPMENT:	3,000
SUBTOTAL:	41,095
INDIRECT COSTS:	6,400
TOTAL:	**47,495**

JUSTIFICATION OF THE BUDGET

SALARIES: 12% of the P.I.'s institutional salary ($47,187) and 100% of the graduate student's stipend ($14,000) plus fringe.

TRAVEL: The P.I. and graduate student presented at three national conferences (6th Psychoneuroimmunology Research Society Conference, Key Biscayne, FL; 55th Annual CPDD Conference, Scottsdale, AZ and 9th International Congress of Immunology, San Francisco, CA) studies that were supported by The Department of Defense.

OPERATING SERVICES: Supports the service contract (with Beckman Instruments) for the scintillation counter that is required for some of the work outlined in this project.

SUPPLIES: Supports the purchase of all reagents and animals as well as the per diem for the animals.

EQUIPMENT: No equipment has been purchased this year. This money is beng redistributed to supplies so that additional experiments can be carried out.

NOTE: This project was originally assigned a budget of $80,684. However, the original P.I. (Dr. Bryan Gebhardt) and the program project director (Dr. Nicolas Bazan) graciously agreed to divide this project in half. However, this decision does not reflect any disagreement or incapatability between parties. Rather, it allows Dr. Gebhardt (P.I. on project 6A) and Dr. Carr (P.I. on project 6B) to focus more closely on their expertise and goals of each specific aim. Moreover, Drs. Carr & Gebhardt interact on a weekly basis over the project, data collection, and analysis of data.

Due to the workload of this project, an undergraduate student (Ms Gina Schilleci) was hired ($5.00/hr) to assist the graduate student (Bill Halford) with the viral infections, plaque assay, virus titers, and tissue culture. Furthermore, due to the relatively small budget, Dr. Gebhardt incurred some of the expenses for animal husbandry on this part (6A) of the project.

PERSONNEL

DANIEL JJ CARR, PH.D.	PRINCIPAL INVESTIGATOR
WILLIAM P. HALFORD, M.S.	GRADUATE STUDENT
GINA SCHILLECI	UNDERGRADUATE STUDENT

Dr. Carr is responsible for the overall organization of this project. Dr. Carr oversees the progress of the graduate student and helps plan and interpret experiments and experimental designs.

Bill Halford is responsible for the all facets of the work associated with this project. Consequently, he infects and screens mice for HSV-1, processes the tissue, cultures latently-infected cells, carries out the RT-PCR for viral and cytokine transcripts, and packages the results for statistical analysis. This project will be the major part of Mr. Halford's dissertation.

Ginal Schilleci assists Mr. Halford in his every day duties as well as organizes and restocks supplies needed in the lab for this project.

ANIMAL USE
1 OCTOBER, 1994, THROUGH JULY 1995

DAMD17-93-V-3013

The experimental animals used during this period for the project, Neural Responses to Injury: Prevention, Protection, and Repair, subproject: **Neuroimmunology of Stress, Injury, and Infection**, are as follows:

Species	Number of animals allowed	Number used	LSU IACUC #
Mouse	200	160	1257

**It should be noted that additional mice (app. 240) were used under a IACUC protocol provided by Dr. Gebhardt (P.I. under chapter 6A).

OTHER SUPPORT

During this year, Dr. Carr was awarded two other small grants:

Ladies Leukemia League: "Molecular Studies on Transcriptional Factors: Association with Leukemia." May 15, 1994 - July 31, 1995. $20,959 total costs. The study investigated the levels of transcriptional factors along with cytokines in splenic lymphocytes obtained from TAT72-transgenic and non-transgenic mice.

LSU Neuroscience Center. "Assessment of the Immune Response to HSV-1 Reactivation in HSV-1 Latently-Infected Mice: In Vitro Correlate." April 3, 1995 - June 30, 1995. $10,000 total costs. The study was centered on developing an *in vitro* corollary for HSV-1 reactivation. The study complimented work progressing from the DoD-supported grant.

SPECIFIC AIMS

**Each original specific aim will be listed and progress on each will be presented along with the significance and future plans.

SPECIFIC AIM #1: **To determine the effects of a brief period of thermal stress (10 min at 43^{o}C) and restraint stress (60 min) as indirect mediators of HSV-1 reactivation from neural tissues.**

A. Determination of viral reactivation in co-culture. These experiments were designed to allow us to determine the frequency of viral reactivation following moderate stressful events and to establish the baseline from which future experiments could be planned. The results presented in the first year's progress report (Tables 1 & 2) showed that the heat stress paradigm induced a

higher percentage of reactivations (80% or 8/10 mice reactivated) as compared to the restraint stress model (40% or 4/10 mice reactivated) as determined by the recovery of infectious virus in the trigeminal ganglia, corneas, and tear film. This experiment was repeated three times with similar results each time. It was noted that statistical analysis of the data indicated a high degree of significance ($p<.005$) as determined by ANOVA. In mice that were not stressed, no recovery of infectious virus was found in any tissue examined. In both cases, the mice were ocularly infected followed by a 35 day incubation period.

B. Immunohistochemical detection of viral reactivation in neural tissue. No experiments have thus far been carried out to immunohistochemically define viral reactivation. However, in the upcoming year, studies will be initiated to section trigeminal ganglia 24-72 hr post heat stress in virally-infected mice and immunohistochemically define the presence of HSV-1 in the trigeminal ganglia using anti-HSV-1 antisera (DAKO).

C. Use of nucleic acid amplification (PCR) to detect stress-induced viral reactivation. We have attempted to use reverse transcriptase (RT)-polymerase chain reaction (PCR) to detect HSV-1 lytic phase transcripts of all three kinetic classes (i.e., immediate-early, delayed-early, and late) as a marker of HSV-1 reactivation following hypothermic stress of latently-infected mice. The HSV-1 specific primers chosen amplify infected cell polypeptide 27 mRNA (i.e., ICP27, immediate early), ribonucleotide reductase mRNA (i.e., RR, delayed-early), and virion protein 23 mRNA (i.e., MP23, late). The ICP27 and VP23 PCR primers have been used before to detect HSV-1 mRNAs during reactivation in a rabbit model (Bloom et al., 1994). However, we have found that aside from a single experiment where ICP27 mRNA was detected in 2/2 mice (115 days post infection) 12 hours after hyperthermic stress, detection of HSV-1 lytic phase transcripts following hyperthermic stress has been inconsistent (2/16 mice) (see Appendix, Fig. 1).

One notable observation that has arisen out of efforts to detect HSV-1 lytic phase transcripts as markers of reactivation is the detecton of VP23 mRNA in the TG of 16/16 latently-infected mice (i.e., 30-44 days post infection), regardless of stress treatment. The identity of these PCR products has been confirmed by Southern blotting (see Appendix, Fig. 2). The possibility that the VP23 PCR product was amplified from contaiminating HSV-1 DNA in the RNA preparation has not been formally ruled out. However, this is unlikely since screening the RT products generated from the mRNA for ICP27 did not detect product. The presence of a HSV-1 RNA species other than the latency associated transcript (LAT) is of interest because there are no known latency-associated proteins, and such a a protein may potentially play an important role in the latent phase of the HSV-1 life cycle.

Significance: The stressors chosen for use in these investigations are bonafide methods of inducing viral reactivation. We have subsequently chosen to employ the heat stress paradigm in all future experiements.

SPECIFIC AIM #2: **To determine the neuroendocrine mechanism of stress-induced viral reactivation by measuring corticosterone and monoamine levels in the serum and nervous tissues of latently infected, stressed animals.**

The goal of this specific aim was to initiate studies in quantitating tissue monoamine and serum corticosterone levels in the stressed mice (infected and uninfected) to determine if a correlation exists between those levels and viral reactivation. In the progress report for year 1 (last year), we presented data measuring serum corticosterone and brainstem monoamine levels 24-hr following hyperthermic stress. Even though this time point is not ideal for such measurements (since neurotransmitter and HPA axis hormone release are rapid responders to stress), the results show that the procedure in isolating and processing tissue allow us to make accurate measurements. We found no differences in the monoamine levels measured (norepinephrine, epinephrine, dopamine, and serotonin) in the brainstem. Serum corticosterone levels were elevated in the stressed mice compared to the non-stressed infected mice. However, there were no differences between serum corticosterone levels between stressed and non-stressed uninfected mice. Potentially, the virally-infected animals which are in a process of viral reactivation may have signalled immune cells to secrete ACTH which could then activate the HPA axis ultimately resulting in an elevation in corticosterone production. Precedence for the involvement of leukocytes in the HPA axis has previously been reported (Blalock, 1984).

Significance: Based on the preliminary studies, we feel confident that accurate measurment can be obtained using both HPLC and RIA to measure monoamines and corticosterone respectively. Consequently, future plans are to undertake a time course study sampling tissues (trigeminal ganglia and serum) from HSV-1 infected and non-infected mice that have or have not undergone hyperthermic stress. Time points that will be taken include 0 min, 30 min, 60 min, 6 hrs, and 12 hrs post-stress episode. Measurements for monoamines and corticosterone will be taken. It is anticipated that these results will be reported in next year's progress report. We currently would like to focus our effort under aims #3 and #4.

SPECIFIC AIM #3: **To determine the effect of stress on antiviral immune responses in mice undergoing viral reactivation.**

A. Serum antibody levels in control and stressed mice. Mice rendered latent for HSV-1 (we use 35 days post-infection) were planned on being sacrificed immediately after stress and at 4, 24, and 96 hr post stress. Antibodies for HSV-1 found in the serum were to be measured by ELISA as described (Gebhardt & Hill, 1988). Dr. Gebhardt (P.I., chapter 6A) has made significant headway in this area.

B. The effect of stress-induced viral reactivation on the CTL reactivity and interferon (IFN)-γ production in mice. In this aim, the splenic lymphocytes from mice sacrificed under Aim #3A will be assayed for CTL activity and IFN-γ production using ^{51}Cr-labeled fibroblasts. We have currently not performed any experiments to measure CTL activity nor IFN-γ production by splenic or lymph node lymphocytes. However, the lab has the capacity of measuring both and preliminary experiments are planned to undertake this investigation in the next fiscal cycle. Preliminary studies indicate that splenic lympphocytes obtained from HSV-1 latently-infected mice produce no measurable IFN-γ but rather IL-2, IL-6, & IL-10 compared to splenic lymphocytes from naive mice when stimulated with HSV-1-infected TGs *in vitro* (data not shown).

C. Cytokine expression in stressed mice during viral reactivation. The goal of this experiment is to investigate the effect of stress on the expression of cytokine genes in the neural tissues and spleens of HSV-1 latently-infected mice. The major amount of time and energy this past year has been devoted to this particular aim. In the experimental design, we investigated the levels of mRNA for proinflammatory cytokines (i.e., IL-1α, IL-6, and TNF-α). In addition, we have also investigated the mRNA levels for other cytokines including IFN-γ and IL-10 as well as the T cell-derived chemokine RANTES. The measurement of mRNA was via reverse transcription-polymerase chain reaction (RT-PCR) to order to insure a sensitive method of detection (more sensitive than a Northern for example). In these experiments, RT-PCR products were obtained from isolating RNA from the trigeminal ganglia of latently infected (INF) or uninfected (UI) mice at time points (t=) 0, 12-, and 24-hrs post hyperthermic stress.

The results shown in Figure 3 (see Appendix, Fig. 3) demonstrate the effects of HSV-1 infection and hyperthermic stress on the cytokine transcription profile within the trigeminal ganglia (TG; i.e. site of viral reactivation). Figure 3 shows two control amplifications which we perform on cDNA samples. Amplification for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA serves as a control for the quality of the cDNA preparation (Fig. 3a). Amplification for the HSV-1 latency-associated transcript (LAT) verifies that each mouse is latently infected (Fig. 3b). The RT-PCR samples shown in these two gel photographs, as well as the other six gel photographs in Figure 4, were amplified from TG cDNA of the same 9 latently infected mice and the same 8 uninfected mice.

Transcription of pro-inflammatory cytokine mRNAs in TG:
Effects of latent HSV-1 infection and hyperthermic stress

The results of RT-PCR comparison of pro-inflammatory cytokine mRNA transcription in the TG of latently infected and uninfected mice (see Appendix, Fig. 4) reveals some interesting differences. Thirty to forty days post-inoculation (PI), IL-1α mRNA expression in the TG is elevated in approximately one-third of latently infected mice, but in the other two-thirds is expressed at the same basal level as uninfected mice (Fig. 4a). While all TNF-α mRNA detected by RT-PCR in latently infected mouse TG is fully spliced, a significantly larger fraction of incompletely spliced TNF-α mRNA is found in uninfected TG (Fig. 4b). Given that TNF-α expression is largely regulated at the post-transcriptional level, this is a potentially significant finding whose relevance needs to be further explored. Hyperthermic stress appears to transiently upregulate IL-6 transcription in the TG, such that IL-6 mRNA levels are elevated at 12 h post stress, but have returned to basal levels by 24 h post stress (Fig. 4c). The hyperthermic stress-induced IL-6 mRNA profile in the TG appears similar in both infected and uninfected mice. The significance of this observation to HSV-1 reactivation is unclear at this point, but clearly demonstrates that the hyperthermic stress model we are using has measurable physiologic effects.

Transcription of T lymphocyte-associated cytokine mRNAs in TG:
Effects of latent HSV-1 infection and hyperthermic stress

RT-PCR comparison of T lymphocyte-associated cytokine mRNA transcription in latently infected TG and uninfected TG indicates that increased transcription of IFN-γ, IL-10, and

RANTES mRNA occurs during latent HSV-1 infection (Figure 4d-f). While there are clear differences between the TG of latently infected and uninfected mice, neither IFN-γ (Fig. 4d), IL-10 (Fig. 4e), nor RANTES (Fig. 4f) mRNA levels in the TG appear to be affected by hyperthermic stress. The infected group of mice whose RT-PCR profile is shown in Fig. 3 were sacrificed 30 days PI. Elevated levels of T cell-associated cytokine mRNA have also been observed in other experiments using mice sacrificed 39 days PI (Fig. 6), 44 days PI (Figure 8), and 115 days PI (data not shown).

These results strongly indicate an association between latent HSV-1 infection and elevated levels of IFN-γ, IL-10, and RANTES mRNAs in the TG. Therefore, it appears that increased numbers of T lymphocytes remain in the TG well after the resolution of acute HSV-1 infection. In order to more fully elucidate the differences in the levels of mRNA for the cytokines that have been tested and found to have some relevance to HSV-1 infection and potentially latency, we propose to use competitive RT-PCR to quantitate differences.

In order to accomplish competitive RT-PCR, we designed a series of mimetics as well as primers that could be used with the mimetics to generate cDNA that could effectively compete with our unknown cDNA templete (generated by RT from the extracted mRNA). We have successfully been able to test the competitive RT-PCR for IL-6, IL-10, and IFN-γ (see Appendix, Fig. 5). Accordingly, we are now in a position to quantitate these cytokine levels in the trigeminal ganglia of the infected and un-infected mice with or without stress.

Based on the hypothesis that infiltrating T lymphocytes are responsible for the observed increase in IFN-γ, IL-10, and RANTES mRNA in latently infected trigeminal ganglia (TG), we have made preliminary attempts at depleting T cells in latently infected mice in order to address the following two questions:

1) Can systemic T cell depletion be utilized to decrease the amount of IFN-γ, IL-10, and RANTES (and their respective mRNAs) in the TG of latently infected mice?
2) Will T cell depletion increase the susceptibility of latently infected mice to HSV-1 reactivation?

In our first preliminary experiment, latently infected mice received 3 subsequent daily i.p. injections of rabbit anti-mouse T cell polyclonal antiserum (i.e. samples D45 and 46) or phosphate-buffered saline (PBS; samples V45 and V46). RT-PCR comparison of TG cytokine profiles in the two treatment groups revealed no obvious differences in IFN-γ, IL-10, or RANTES mRNA expression (see Appendix, Fig. 6). FACS analysis of splenic lymphocytes confirmed that there was a depletion in $CD8^+$ lymphocytes (see Appendix, Fig. 7) in mice receiving anti-T cell antiserum relative to PBS-injected controls, however, no such decrease was observed in $CD4^+$ lymphocytes.

In a second T cell depletion experiment, latently infected mice treated with either anti-T cell antiserum (i.e. sample D47) or PBS (i.e. samples V47 and V48) were hyperthermically stressed, and sacrificed 24 hours after the stressor. Levels of IFN-γ, IL-10, and RANTES mRNA appeared to be lower in the antiserum-injected/ heat-stressed mouse relative to PBS-injected / heat-stressed controls (see Appendix, Fig. 8). While the results are encouraging, more work is required.

Significance: Based on these preliminary results reported above and reproduced again for an n value of 5/group tested, we feel confident that further exploration in determining the levels of some of these cytokines will bear fruition to the relationship between cytokine levels and viral reactivation. To this end, we plan on undertaking competitive RT-PCR for the quantitative determination of mRNA extracted from the trigeminal ganglia at different time points (4-24 hrs) (of HSV-1 infected and un-infected mice with or without stress) encoding IL-6, IL-10, IFN-γ, and the T cell-derived chemokine, RANTES. In addition, protein levels for these cytokines in the trigeminal ganglia will also be determined by ELISA (IL-6, IL-10, and IFN-γ are routinely measured in this lab) or immunohistochemically (RANTES). The results of these experiments will be used along with a series of planned experiments using an *in vitro* HSV-1 reactivation system to fully elucidate the role of cytokines in HSV-1 reactivation or hindrance of reactivation following stress.

Relative to defining T cell subsets that may be involved in the regulatory effects of HSV-1 reactivation following stress, work is planned to deplete initially all T cells ($CD3^+$, $CD4^+$, and $CD8^+$) and subsequently deplete selective subpopulations to determine if one or more of the populations is responsible for the cytokine mRNA expression in the TG.

SPECIFIC AIM #4: **To determine the effect of exogenous stress hormone agonists and antagonists on stress-induced viral and antiviral immunity.**

The hypothesis on which this investigation is based centers on the notion that corticosterone and/or catecholamines are in part responsible for the reactivation of HSV-1 in latently infected mice following hyperthermic stress. Accordingly, the administration of exogenous catecholamines or corticosteroids are predicted to reactivate HSV-1 without subjecting the latently infected animals to the stress paradigm.

Progress has been made in this aim using both *in vivo* and *in vitro* systems. Specifically, the administration of propranolol (β-adrenoceptor antagonist) has been shown to suppress the reactivation of HSV-1 latently-infected mice (Gebhardt & Kaufman, in press, see chapter 6A). These results would suggest that catecholamines may play a significant role in HSV-1 reactivation. Of course, future experiments will be required to show a dose-dependent effect as well as to differentiate between β_1 and β_2 mediated pathways using selective agonists and antagonists. However, this is the first reported case showing a direct cause/effect with adrenergic antagonists.

Likewise, our lab has utilized a dissociated TG culture paradigm of HSV-1 reactivation described by Moriya, et al. (1994) to test biological mediators for their ability to induce HSV-1 reactivation from latently-infected neurons. We have demonstrated that latent HSV-1 infection is maintained in these TG cultures by 1) detecting LAT mRNA in 10/10 dissociated TG monolayers ten days after plating (data not shown), and 2) by inducing efficient reactivation from these cultures with a hyperthermic stressor delivered 15 days after plating ranging from 70-90% reactivation of latently-infected neural cultures.

Since it was apparent the *in vitro* culture system paralled the *in vivo* hypothermic stress mouse model reactivation of latent HSV-1, a series of experiments were carried out to determine the

direct role of hormones and predicted association with reactivation of latent HSV-1 in the TGs. Latent HSV-1 did not reactivate from this dissociated TG culture system in response to any of the following drug treatments (n=8-14 wells/drug repeated at least twice): 10^{-6} M, 10^{-8} M, 10^{-10} M epinephrine; 50 μM and .5 μM forskolin, 500 μM chlorophenylthio-cAMP, and 10^{-7} M morphine (data not shown). The presence of latent, reactivatable HSV-1 in these TG cultures was confirmed after drug treatment by heat shock-induced reactivation (70-90 % reactivated). Therefore, while direct roles for epinephrine and cAMP have been postulated in stress-induced HSV-1 reactivation (Hill, et al., 1987; Smith et al., 1992), we find that neither of these mediators by themselves induce HSV-1 reactivation from dissociated, latently infected TG cultures.

As a correlate of our *in vivo* studies, we have attempted to measure the effect of hyperthermic stress (i.e. same treatment used to reactivate latent HSV-1 in this model) on cytokine production from both latently infected and uninfected dissociated TG cultures. We have found no detectable amounts of IL-10 (less than 50 pg/ml) IFN-γ, or TNF-α concentrations as determined by ELISA (with a sensitivity range down to 50 pg/ml for IL-10 and TNF-α and 30 Units/ml for IFN-γ). However, IL-6 is readily detectable in latently infected and uninfected TG cultures. We have found that 24 hours after heat stress, latently-infected TG cultures contain modestly elevated IL-6 concentrations while uninfected TG cultures contain significantly less IL-6 (Table I). Notably, our *in vivo* RT-PCR results suggest that a similar pattern of IL-6 induction occurs in latently infected mice following hyperthermic stress (Fig. 4c).

TABLE 1. HYPERTHERMIC STESS CHANGES IL-6 PRODUCTION BY TG CULTURED NEURONS[a]

HSV-1 INFECTED	Prior to hyperthermic stress	24-HR post hyperthermic stress	48-HR post hyperthermic stress	72-HR post hyperthermic stress
-	63.5 +/- 6.7[b]	37.7 +/- 5.3*	50.9 +/- 6.8	28.6 +/- 3.5**
+	64.0 +/- 7.9	80.2 +/- 7.6	52.5 +/- 4.4	41.6 +/- 4.6*

[a]TG neuron cultures were initiated from surgically removing the TGs from latently-infected ICR mice and processing the neurons as described (Moriya et al., 1994). HSV-1 reactivation has been monitored in each processed TG preparation (typically 10 TGs per 24-well microtiter plate). Typically, the processed TGs are placed in culture with or without (positive control) (E)-5-(2-bromovinyl)-2-deoxy-uridine (5-BVDU5 μg/ml). The concentration of 5-BVDU used in the cultures was emperically determined in our lab using HSV-1-latently infected TGs testing concentrations ranging from 0.5-500 μg/ml of 5-BVDU. After 10-12 days in culture, the HSV-1 latently-infected TG supernates were sampled and TG cultures were subsequently stressed (43° C for 180 min). Supernate samples were collected at 24, 48, and 72 hrs post stress and measured for IL-6.
[b]Numbers (ng/ml) are the mean +/- SEM, n=24/group as determined by ELISA. This table is the summary of 3 independent experiments using 8 wells per-infected or uninfected TG groupings/experiment. **$p<.01$, *$p<.05$ comparing the IL-6 levels post stress to pre-stress levels in both infected and uninfected TG cultures. There was no detectable IL-2, IL-10, or IFN-γ in any of the culture supernates tested (data not shown).

In parallel to the above mentioned studies investigating stress-induced reactivation of HSV-1 latently infected mice, the laboratory has been involved in investigating chemical stressors that might be involved in the pathogenic processes of HSV-1 infection or reactivation as well. Studies conducted by Holaday et al. while working in the Division of Neuropsychiatry of Walter Reed Army Institute provided evidence that morphine is a potent immunosuppresssive drug when

administered *in vivo*. The immunosuppression observed following morphine adminisntration suggested that T cell-mediated events including lymphocyte proliferation can be severely afftected following morphine exposure (Bryant et al., 1988; Bryant et al., 1991). Based on these observations, a series of studies were carried out to explore the effects of morphine on CTL activity (since these effector cells are central to anti-viral immunity). Four published manuscripts describe various experiments that show morphine suppresses CTL activity through the involvement of opioid receptors classified most closely with μ (see Appendix, manuscipts 1-4). A fifth article reviews the current working model developed in part in this laboratory by which morphine is predicted to suppress CTL activity (see Appendix, manuscript #5). The sixth article describes work in which the P.I. and graduate student have identified the first functional role for an orphan opioid receptor in the immune system (see Appendix, Manuscript #6).

It is anticipated that this work will be directly applicable to the goals of this proposal. Specifically, one of the studies (Carpenter et al., 1994) has shown that morphine acts as a co-factor in potentiating viral-induced encephalomyelitis in HSV-1 acutely-infected mice. Our initial experiments outlined under Aim #4 have suggested that morphine (10^{-7} M) does not alone reactivate latently-infected TGs *in vitro*. These results will be repeated at different doses of morphine (10^{-5} - 10^{-11} M). In addition, morphine will also be used to determine if it acts as a co-factor in hyperthermic stress induction of HSV-1 reactivation similar to what we have found in preliminary results using dexamethasone (10^{-7} - 10^{-11} M) (data not shown). Based on our hypothesis that morphine acts as a co-factor to HSV-1 reactivation as well as pathology (due to its immunosuppressive properties) associated with the acute infection, experiments are planned to determine if morphine alone can reactivate HSV-1 latently-infected mice or synergize with hyperthermic stress in the reactivation of HSV-1 latently-infected mice. The results of these experiments are in line to the mission of the Department of Defense since acute and chronic pain are typically treated using pain relief compounds including opioids. Since Bryant et al (1988) have shown morphine induced the activation of the hypothalamic pituitary adrenal axis production of corticosterone and we have preliminary data indicating the supercorticosterone, dexamethasone potentiates HSV-1 reactivation of latently-infected TGs, we hypothesize that a dynamic relationship will be elucidated between morphine and HSV-1 pathology and reactivation following hyperthermic stress.

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PUBLICATIONS RESULTING FROM THIS RESEARCH:

Publications:

1. Carpenter, G.W., H.H. Garza jr., B.M. Gebhardt, & D.J.J. Carr. 1994. Chronic morphine treatment suppresses CTL-mediated cytolysis, granulation, and cAMP responses to alloantigen. *Brain, Behavior, Immun.* 8:185-203.

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5. Halford, W.P., B.M. Gebhardt, and D.J.J. Carr. 1995. Functional role and sequence analysis of a lymphocyte orphan opioid receptor. *J. Neuroimmunol.* 59:91-101.

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Abstracts:

1. Halford, W.P., B. Gebhardt, and D.J.J. Carr. 1994. HSV-1 latently infected mice display an altered response to stress: Implications for antiviral immunity. 6th Psychoneuroimmunology Research Conference, Nov. 17-20, Key Biscayne, FL

2. Halford, W.P., B.M. Gebhardt, and D.J.J. Carr. 1994. Mouse lymphocytes express an orphan opioid receptor. 6th Psychoneuroimmunology Research Conference, Nov. 17-20, Key Biscayne, FL.

3. Carr, D.J.J., L. Breeden, G.W. Carpenter, & B.M. Gebhardt. 1994. The frequency of exposure to morphine differentially affects CTL activity in alloimmunized mice. 6th Psychoneuroimmunology Research Conference, Nov. 17-20, Key Biscayne, FL.

4. Baker, M.L., D.J.J. Carr, & B.M. Gebhardt. 1994. Morphine suppresses peritoneal and splenic CTL activity in a dose dependent fashion in alloimmunized mice. 6th Psychoneuroimmunology Research Conference, Nov. 17-20, Key Biscayne, FL.

5.. Halford, W.P., M. Serou, B.M. Gebhardt, & D.J.J. Carr. 1995. Functional role and sequence analysis of a lymphocyte orphan opioid receptor. CPDD Meeting, June 10-15, Scottsdale, AZ

6. Halford, W.P., B.M. Gebhardt, and D.J.J. Carr. 1995. Analysis of the immune response during stress-induced reactivation of herpes simplex virus type 1. 9th International Congress of Immunology, July 23-29, San Francisco, CA.

APPENDIX

Item	Content
1.	Figures 1-8
2.	Carpenter et. al., 1994
3.	Carpenter & Carr, 1995
4.	Carr & Carpenter, 1995
5.	Carpenter et al., in press
6.	Carr et. al., 1995
7.	Halford et al., 1995

FIG. 1.

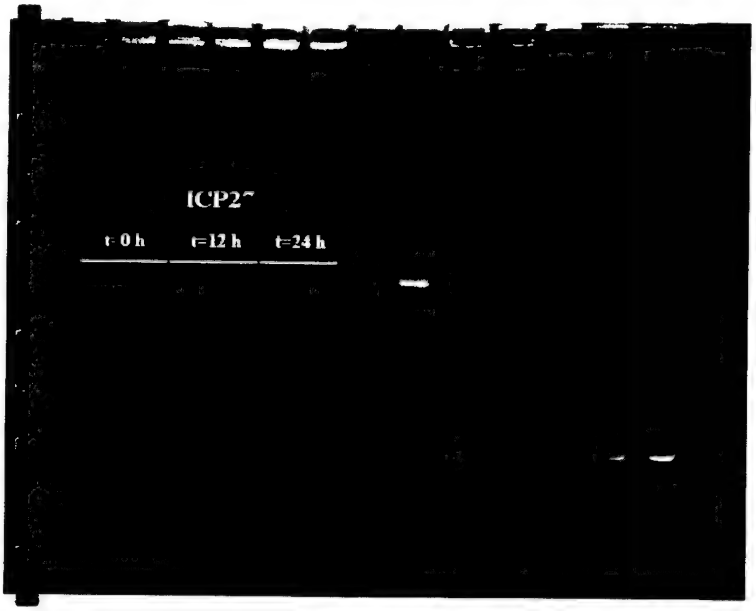


Fig 2

Fig 3a

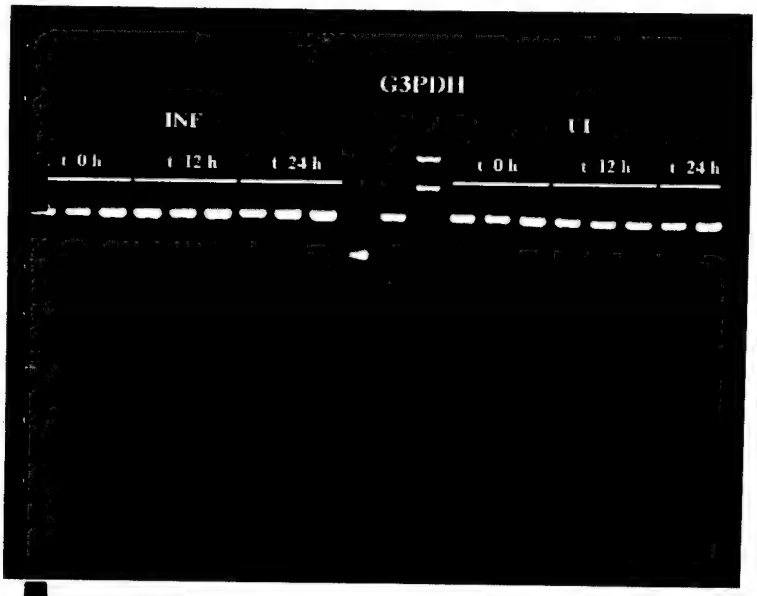


Fig 3b

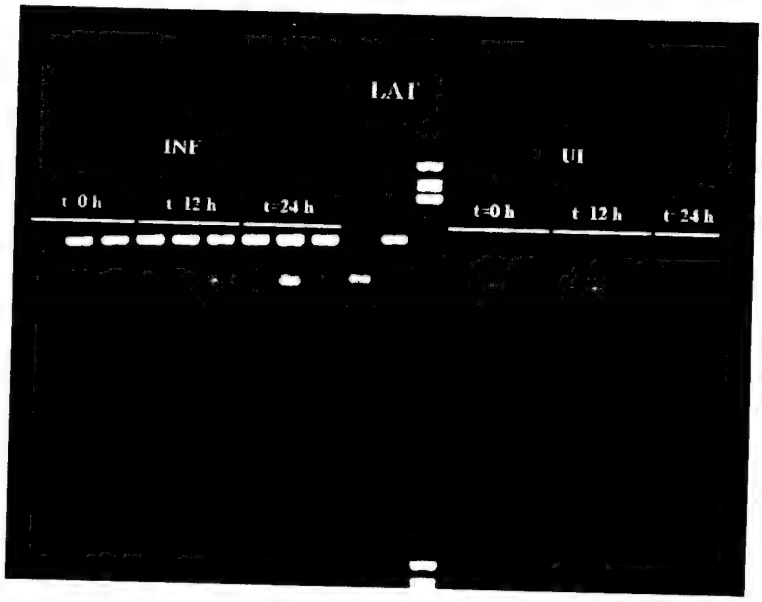
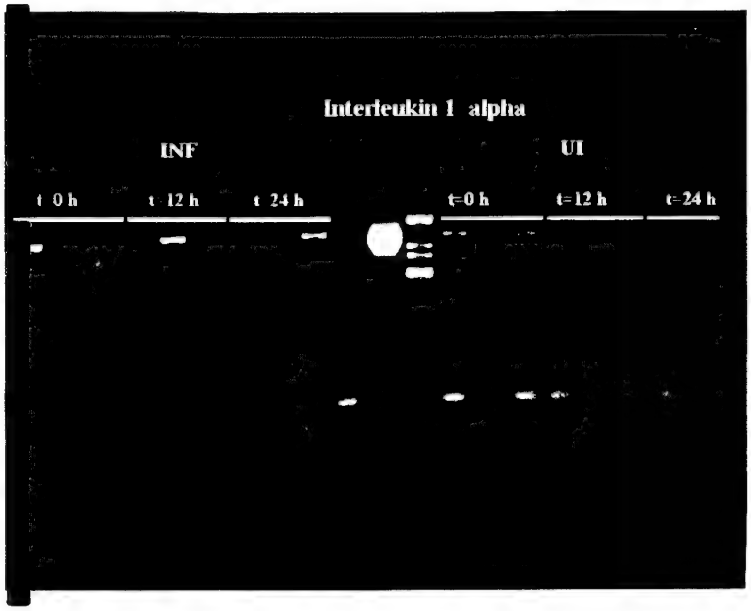


Fig 4a



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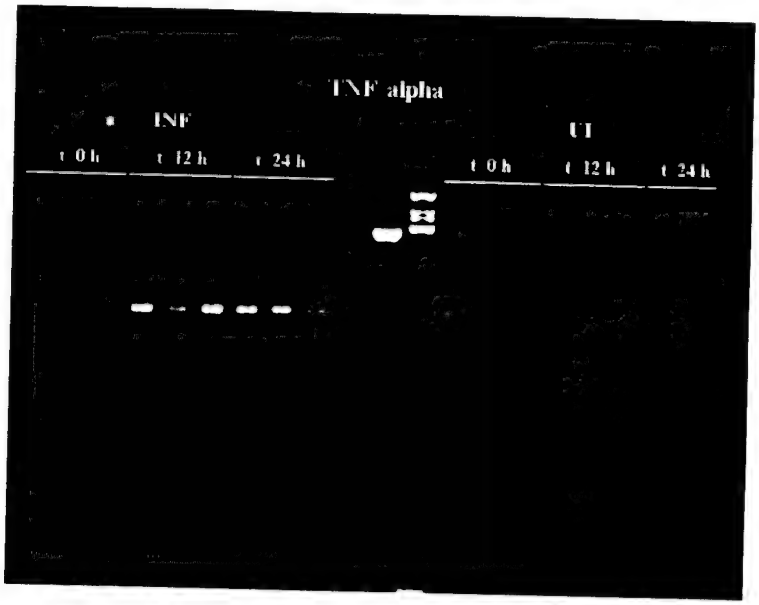

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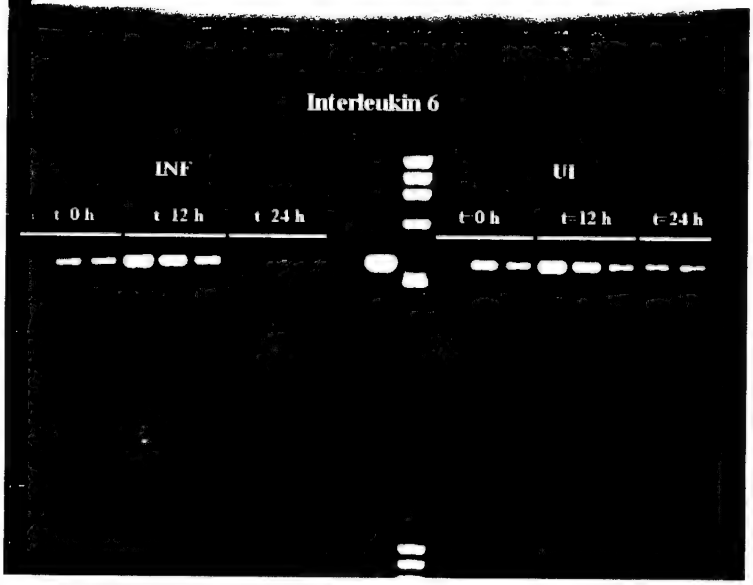

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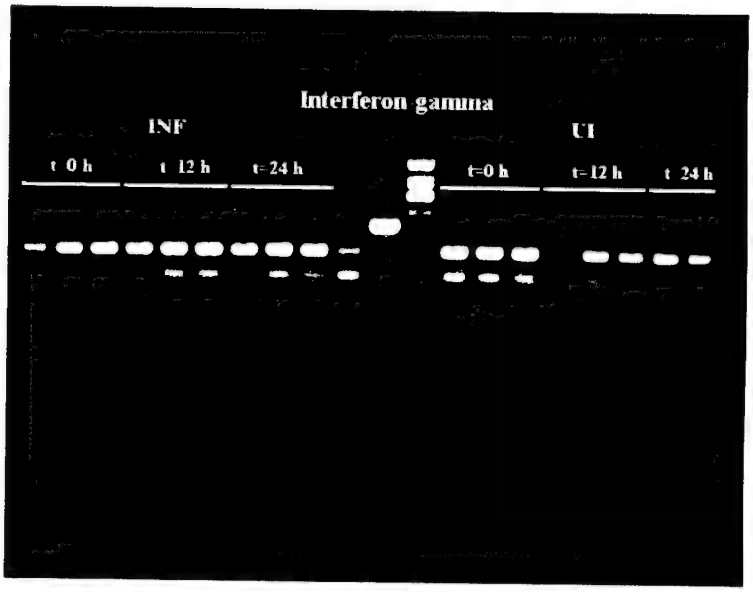
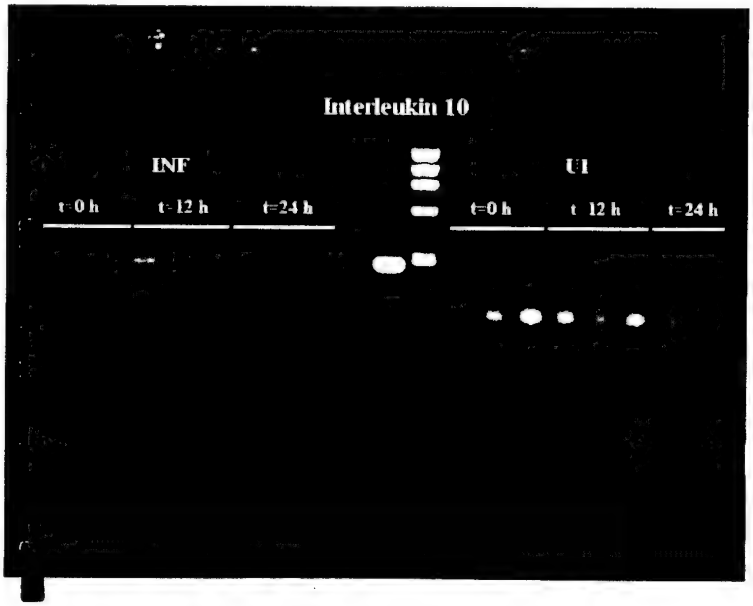


Fig 4e



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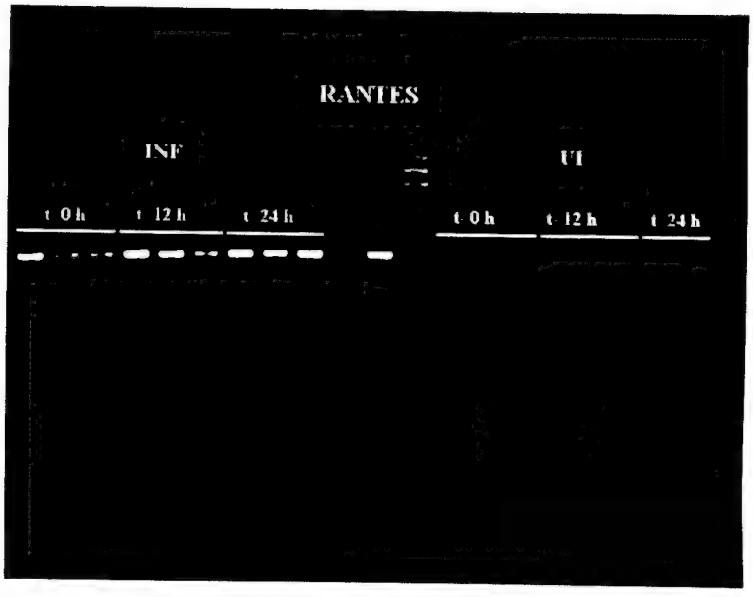


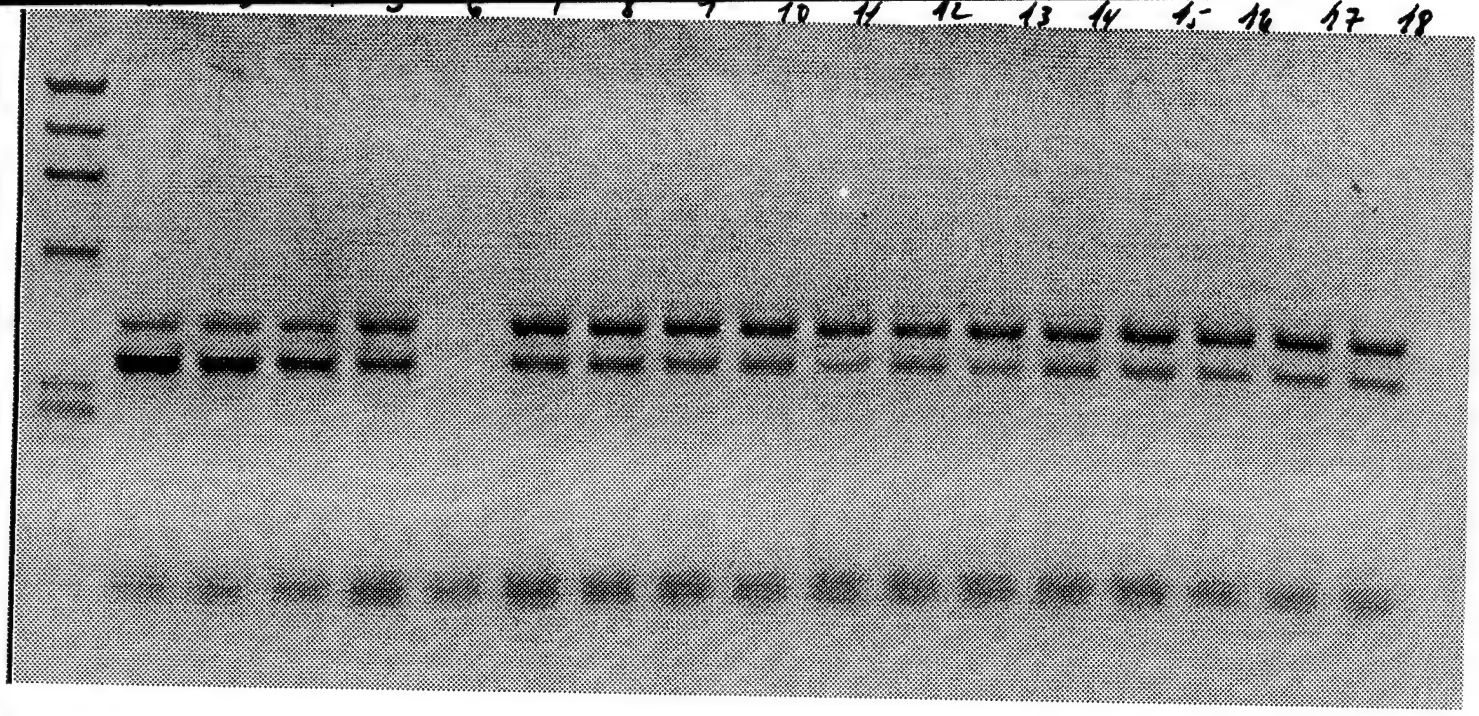


FIGURE 5A. RT-PCR of IL-6 transcript levels obtained from lipopolysaccharide-activated splenic lymphocytes. Total RNA was isolated from 10-40X10^6 splenic lymphocytes stimulated with 50 μg of lipopolysaccharide using Ultraspec (biotecx, Houston, TX). First strand cDNA synthesis was performed on 1 μg aliquots of total RNA using a cDNA synthesis kit purchased from Promega (Madison, WI). RT reactions were carried out at 42° C for 1 hr using the poly(dT) primers provided. Once synthesized, specific cDNAs encoding IL-6 or glyceraldehyde-3-phosphate dehydrogenase were measured using primer competition PCR reactions. A series of PCR reactions were performed on aliquots of cDNA, each spiked with a known amount (12.5-800 copyequivalents) of PCR mimetic in order to create an internal standard curve (lanes 2-5). The PCR mimetic was a larger, internally non-homologous DNA fragment that competed for the same primers that amplified the target cDNA. After PCR amplification, the two species could be separated by agarose gel electrophoresis and analyzed densitometrically. When the cDNA aliquot was amplified to the same intensity as the internal competitive standard, they are termed "copy equivalent." Imagequant analysis can subsequently be used to quantitate the amount of copy equivalents per RT-PCR reactions and thus, per sample. A representative figure for IL-6 is shown above. Lane 1 is the DNA ladder; Lane 6 is the primer controls, and Lanes 7-18 are experimental samples.

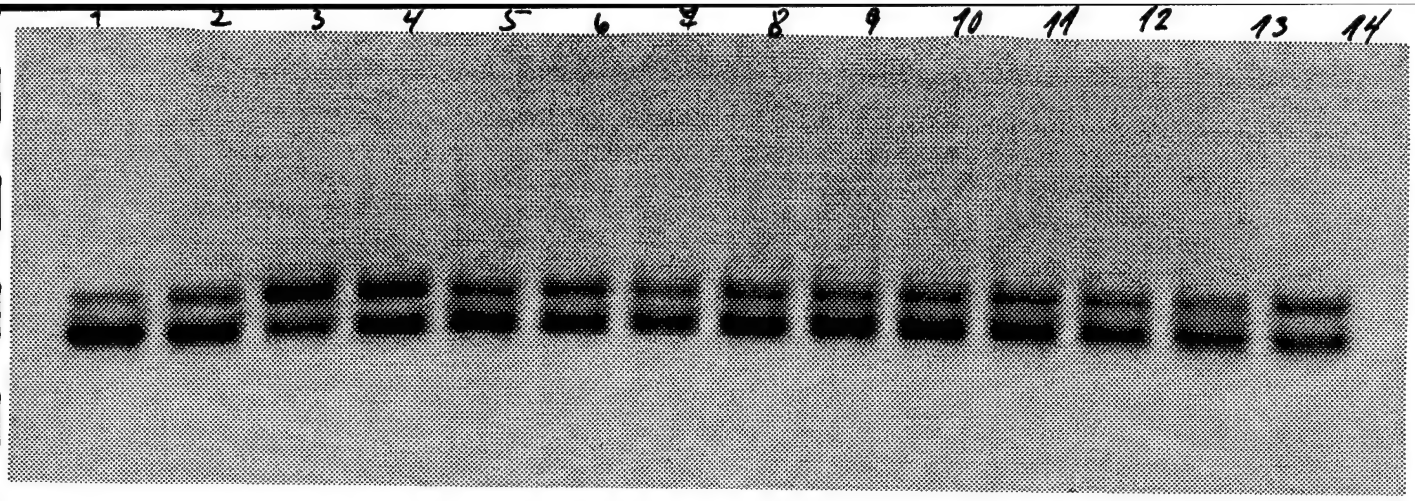


FIGURE 5B. RT-PCR of IL-10 transcript levels obtained from concanavalin A-activated splenic lymphocytes. Total RNA was isolated from 10-40X10^6 splenic lymphocytes stimulated with 75 μg of concanavalin A using Ultraspec (biotecx, Houston, TX). The RT-PCR set-up is identical as indicated in Fig. 5A legend. A representative figure for IL-10 is shown above. Lane 1-4 are the internal standard curve for the cDNA and mimetic of known copy equivalent 800-12.5 copy equivalents. Lanes 5-14 are experimental samples.

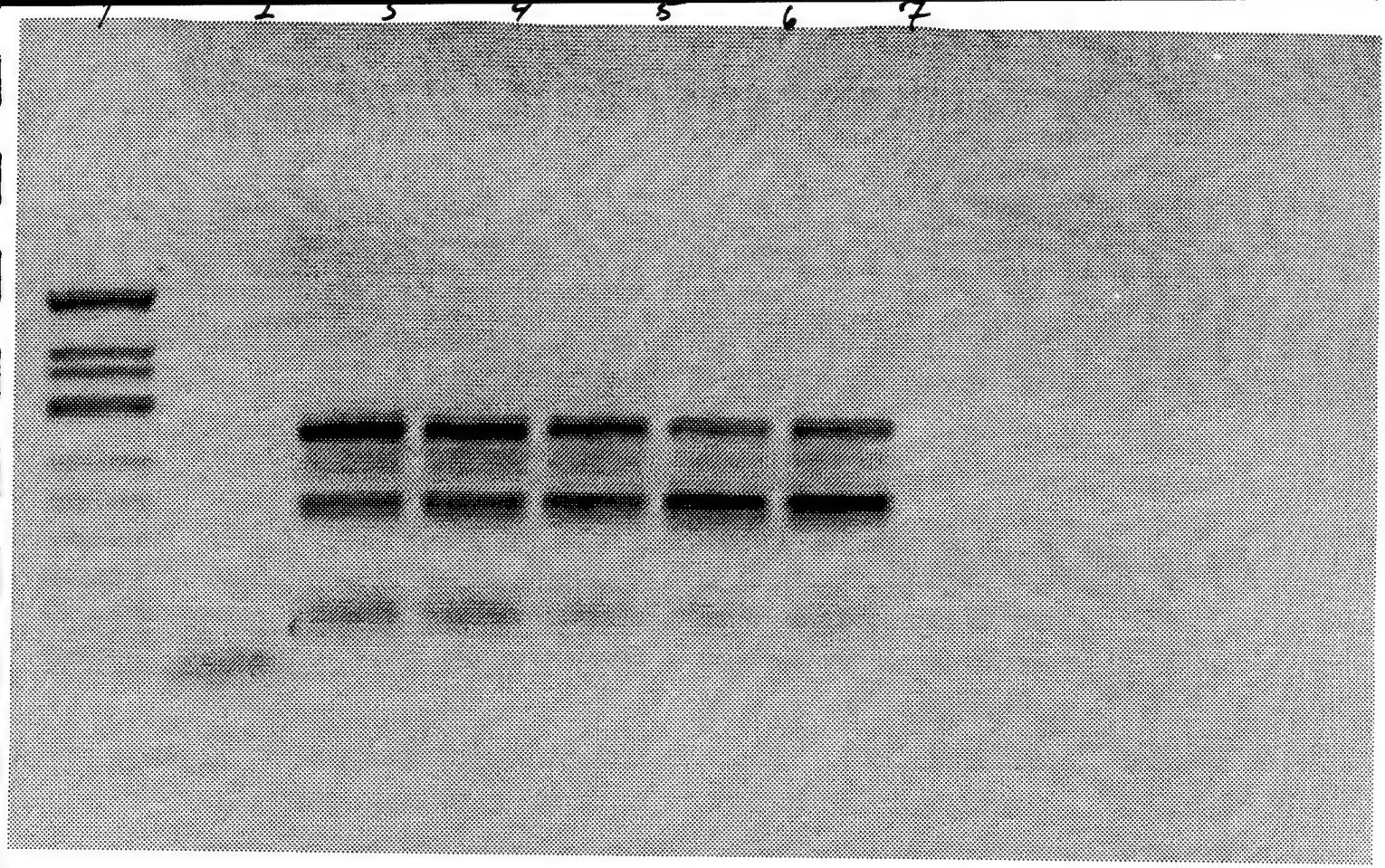


FIGURE 5C. RT-PCR of IFN-γ transcript levels obtained from concanavalin A-activated splenic lymphocytes. Total RNA was isolated from 10-40X10^6 splenic lymphocytes stimulated with 75 μg of concanavalin A using Ultraspec (biotecx, Houston, TX). The RT-PCR set-up is identical as indicated in Fig. 5A legend. A representative figure for IFN-γ is shown above. Lane 1 is the DNA ladder. Lane 2 is the primer control. Lanes 3-7 are the internal standard curve for the cDNA and mimetic of known copy equivalent 800-12.5 copy equivalents. No experimental samples are shown although they have been carried out. In addition, no competitive RT-PCRs are shown for the housekeeping gene G3-PDH although they have been carried out as well. Currently, two manuscripts using this technique and measuring cytokines (IL-2, IL-6, and IL-10) are in preparation for submission by Sept. 1, 1995.

Fig 6

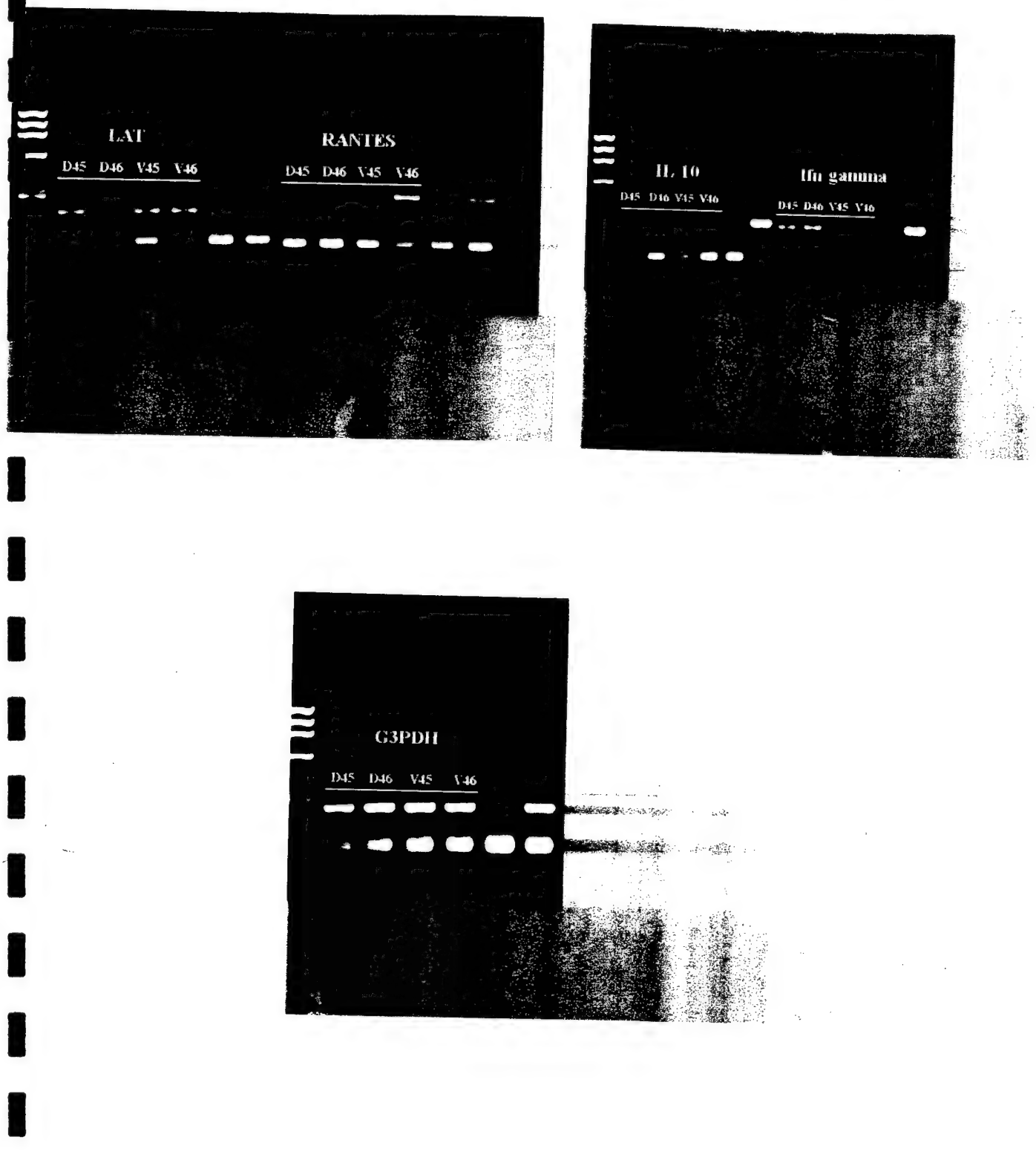
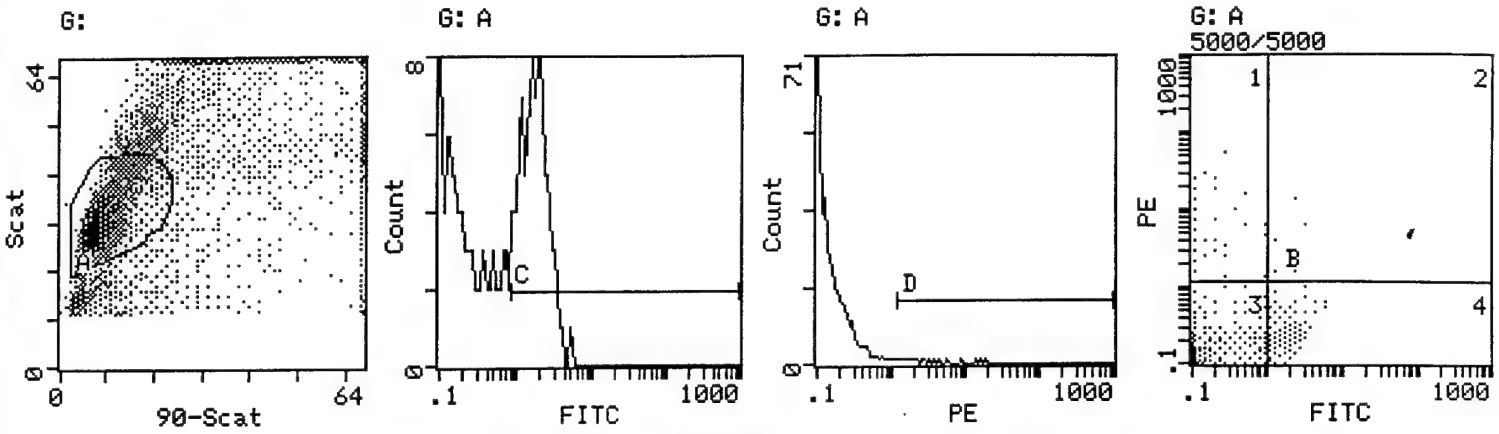

LAT
D45 D46 V45 V46
RANTES
D45 D46 V45 V46
IL 10
D45 D46 V45 V46
Ifn gamma
D45 D46 V45 V46
G3PDH
D45 D46 V45 V46

FIGURE 7. FACS ANALYSIS OF ANTI-T CELL TREATED MICE

FITC = CD4+
PE = CD8+

E. FACS PROFILE OF ANTI-T SERUM (D45)-TREATED MOUSE



SINGLE PARAMETER STATISTICS

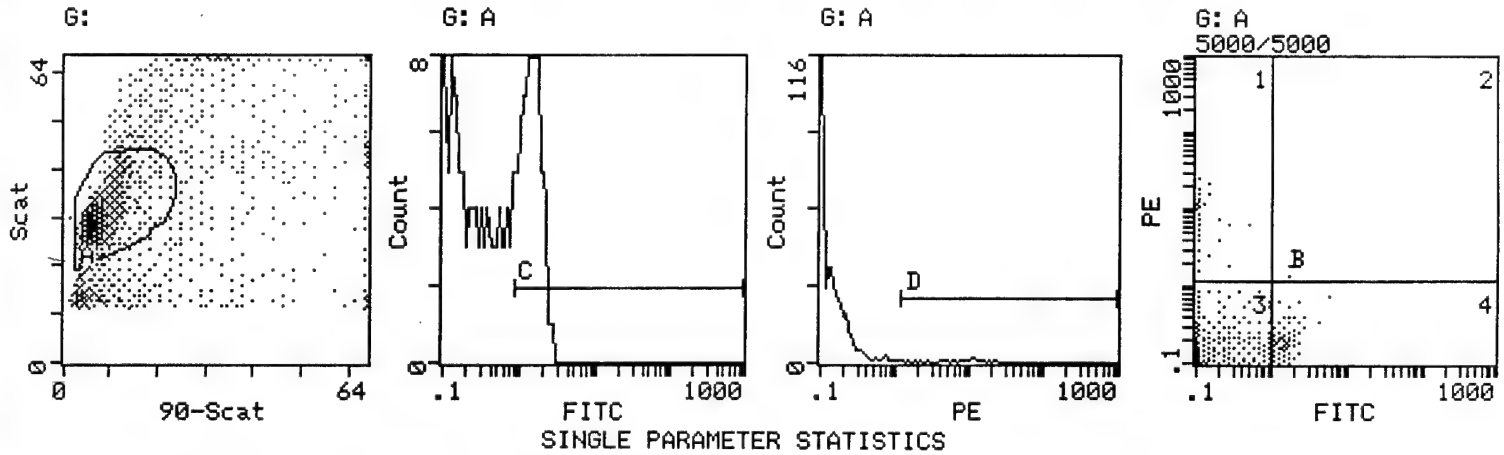
			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	18.4	920	1.9	13	1.77	0.951	53.8	4.13	0.83	1024
D	3.7	186	1.6	4	4.89	5.77	118.0	0.446	1.2	1024

DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	41.2	5000	7, 30	87	10.2	4.3	42.5	31.5	5.9	18.6

F. FACS PROFILE OF ANTI-T SERUM (D46)-TREATED MOUSE

FITC = CD4+
PE = CD8+



SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	15.4	769	1.3	15	1.41	0.538	38.1	2.81	0.83	1024
D	5.3	266	9.0	6	6.66	6.75	101.4	0.420	1.2	1024

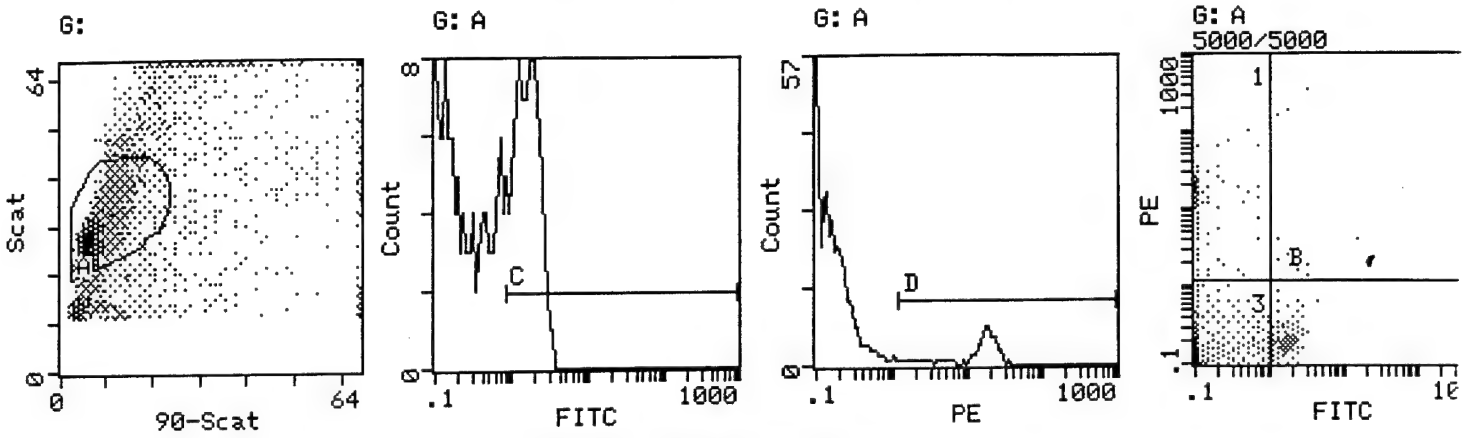
DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	53.7	5000	5, 28	173	8.3	4.0	48.6	29.9	5.1	17.2

FIGURE 7. FACS ANALYSIS OF ANTI-T CELL TREATED MICE

FITC = CD4+
PE = CD8+

C. FACS PROFILE OF VEHICLE (V45)-TREATED MOUSE



SINGLE PARAMETER STATISTICS

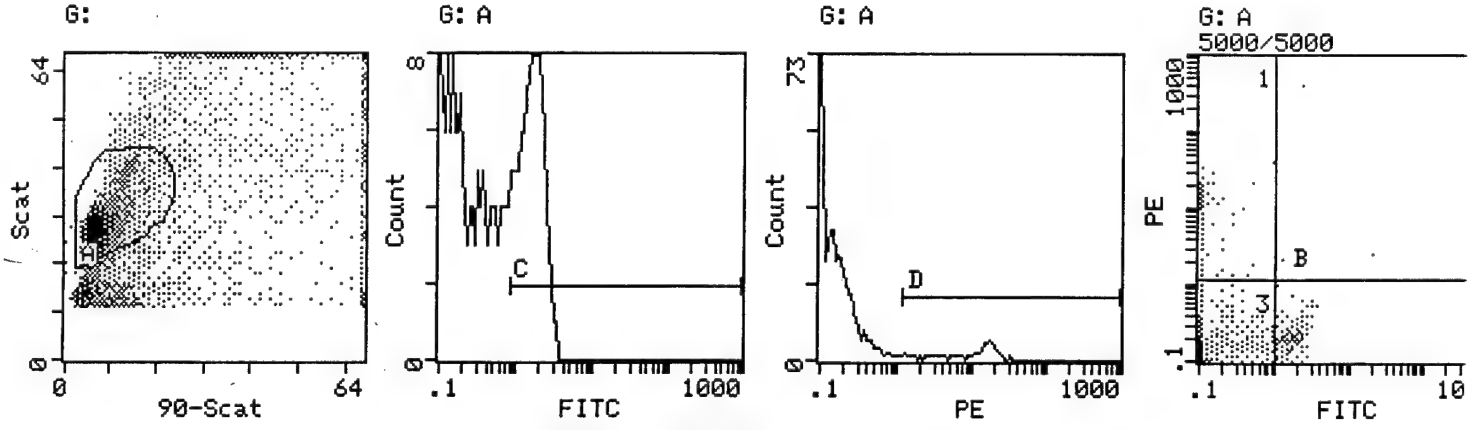
			.....Peak.....		.....X Channel.....					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	19.4	969	1.9	18	1.63	0.666	40.8	0.726	0.83	1024
D	13.6	681	17	13	11.5	11.1	96.2	0.559	1.2	1024

DUAL PARAMETER STATISTICS

			.....Peak.....		....X Channel....			....Y Channel....		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	51.4	5000	5, 26	136	8.8	4.1	46.4	29.5	5.8	19.6

FITC = CD4+
PE = CD8+

D. FACS PROFILE OF VEHICLE (V46)-TREATED MOUSE



SINGLE PARAMETER STATISTICS

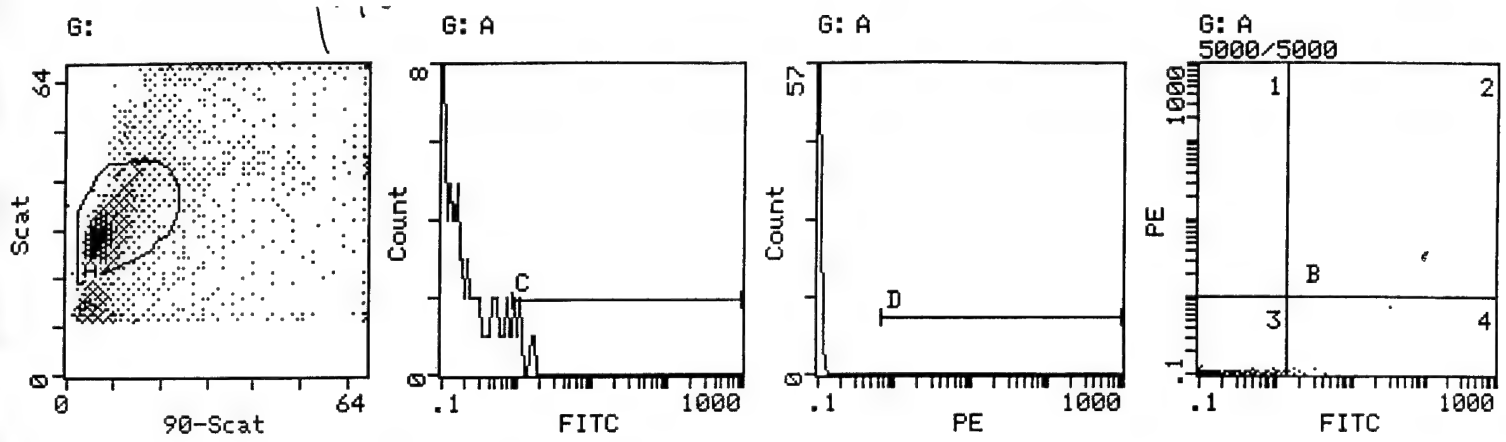
			.....Peak.....		.....X Channel.....					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	18.2	912	1.5	17	1.62	0.677	41.7	0.913	0.83	1024
D	10.1	504	16	9	9.47	9.84	103.9	0.630	1.2	1024

DUAL PARAMETER STATISTICS

			.....Peak.....		....X Channel....			....Y Channel....		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	42.7	5000	5, 26	88	9.6	4.5	46.6	29.4	5.6	19.2

FIGURE 7. FACS ANALYSIS OF ANTI-T CELL TREATED MICE

A. ISOTYPIC CONTROL FLUORESCEIN ISOTHIOCYANATE (FITC)



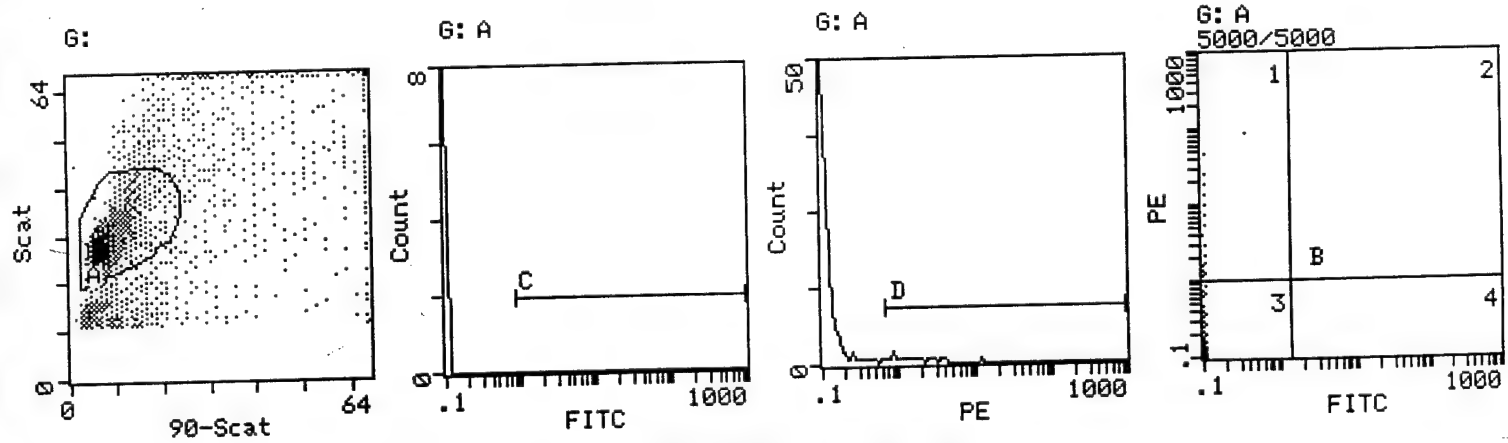
SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	2.3	113	0.90	6	1.51	1.14	75.8	0.573	0.83	1024
D	0.0	2	0.80	1	0.836	0.034	4.05	0.382	0.70	1024

DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	54.0	5000	5, 28	144	8.3	3.9	46.3	29.7	5.0	16.7

B. ISOTYPIC CONTROL PHYCOERYTHRIN (PE)



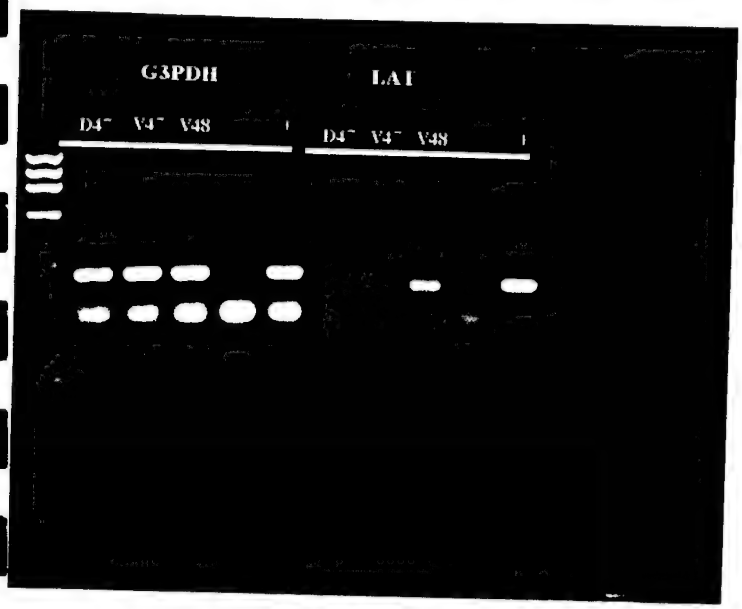
SINGLE PARAMETER STATISTICS

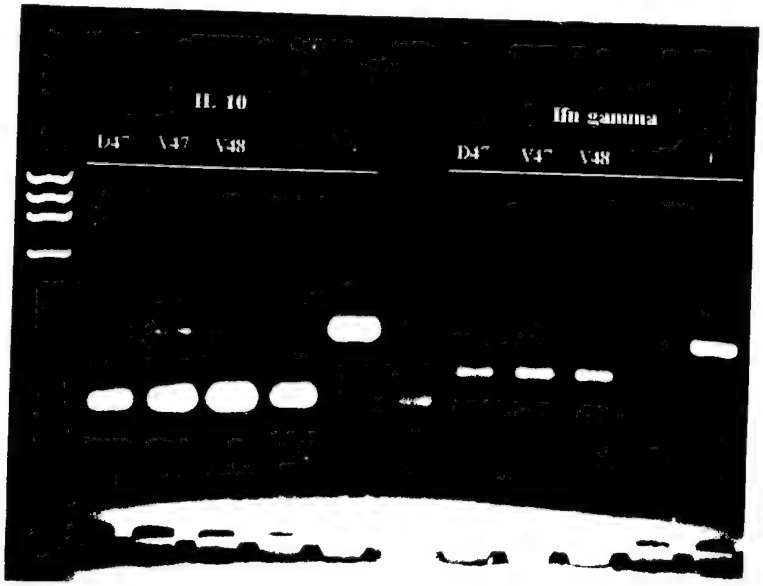
			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	0.1	4	1.2	1	3.35	3.45	102.9	0.382	0.83	1024
D	4.6	230	0.86	4	2.56	3.32	129.8	0.573	0.70	1024

DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	49.6	5000	5, 26	108	9.1	4.3	46.8	29.8	5.5	18.3

Fig 8


G3PDH
LAT
D4
V47
V48
D4
V47
V48


IL 10
Ifn gamma
D4
V47
V48
D4
V47
V48


RANTES
D4
V47
V48

BRAIN, BEHAVIOR, AND IMMUNITY 8, 185–203 (1994)

Chronic Morphine Treatment Suppresses CTL-Mediated Cytolysis, Granulation, and cAMP Responses to Alloantigen[1]

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Departments of Microbiology, Immunology, Parasitology, †Ophthalmology, and §Pharmacology, and ‡LSU Neuroscience Center, Louisiana State University Medical Center, New Orleans, Louisiana 70112-1393

Exposure to opioid drugs (e.g., morphine) *in vivo* has been shown to suppress natural killer cell activity. However, the effects of *in vivo* exposure to opioids on cytotoxic T lymphocyte (CTL) activity has not been investigated. The administration of morphine (50.0 mg/kg, sc) to alloimmunized mice for 11 days resulted in a significant decrease in peritoneal and splenic CTL activity. Moreover, the intracellular content of serine esterases and esterase release by $CD8^+$ effector cells from chronic morphine-treated mice was reduced compared to that of effector cells from vehicle-treated controls. In addition, the $CD8^+$ cAMP response to alloantigen was diminished compared to $CD8^+$-enriched cells from vehicle-treated animals. However, conjugate formation between effector and target and subsequent killing of target by effector cells did not reveal significant differences between vehicle- and chronic morphine-treated animals. Serum corticosterone and dehydroepiandrosterone levels were significantly lower in the chronic morphine-treated animals while proopiomelanocortin gene expression (exon 3) in splenic lymphocytes did not correlate with morphine-mediated suppression of CTL activity. These results indicate that CTL activity is sensitive to chronic morphine exposure, implicating opioids as important cofactors during viral infections in suppressing cell-mediated immunity.

INTRODUCTION

Short-term (<120 h) morphine administration has been shown to reduce natural killer (NK)[3] activity (Shavit, Lewis, Terman, Gale, & Liebeskind, 1984; Bayer, Daussin, Hernandez, & Irvin, 1990), impair immunoglobulin production (Bussiere, Adler, Rogers, & Eisenstein, 1992; Pruett, Han, & Fuchs, 1992), suppress phagocytic activity (Levier, Brown, McCay, Fuchs, Harris, & Munson, 1993; Szabo, Rojivin, Bussiere, Eisenstein, Alder, & Rogers, 1993), and induce thymic

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[3] Abbreviations used: ACTH, adrenocorticotropic hormone; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; CTL, cytotoxic T lymphocyte; DHEA, dehydroepiandrosterone; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunoabsorbent assay; EPI, epinephrine; FACS, Fluorescence activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HBSS, Hank's buffered saline solution; HSV-1, herpes simplex virus type-I; HPA, hypothalamic-pituitary adrenal; ICAM, intracellular adhesion molecule; IFN, interferon; IL, interleukin; ip, intraperitoneal: LU, lytic unit; NE, norepinephrine; NK, natural killer; PEL, peritoneal exudate leukocytes; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLC, phospholipase C; POMC, Proopiomelanocortin; RT, reverse transcription; sc, subcutaneous; SL, splenic lymphocyte; SNS, sympathetic nervous system; Triton X-100, *t*-octylphenoxypolyethoxyethanol.

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hypoplasia (Fuchs & Pruett, 1993). In monkeys and humans, chronic morphine use is known to suppress NK activity (Novick, Ochshorn, Ghali, Croxson, Mercer, Chiorazzi, & Kreek, 1989; Carr & France, 1993).

The action of opioids on the nervous system has been proposed as the route to immunomodulation. Studies have shown that morphine can act centrally (Shavit, Depaulis, Martin, Terman, Pechnick, Zane, Gale, & Liebeskind, 1986) through receptors located in the periaqueductal gray matter of the mesencephalon (Weber & Pert, 1989). Two central nervous system (CNS) pathways have been shown to be involved in opiate-induced immunomodulation, the hypothalamic pituitary adrenal (HPA) axis, and the sympathetic nervous system (SNS). The HPA axis has been implicated in short-term studies of opioid-induced immunosuppression through the release of corticosterone (Bryant et al., 1991; Sei, Yoshimoto, McIntyre, Skolnick, & Arora, 1991; Pruett et al., 1992; Fuchs et al., 1993; Migliorati, Nicoletti, D'Adamio, Spreca, Pagliacci, & Riccardi, 1994). Endogenous corticosterone is selectively immunosuppressive suppressing interleukin (IL)-1, IL-2, and interferon (IFN) gamma production but acting synergistically with γ-IFN in enhancing monocyte activation and the generation of reactive oxygen intermediates (for review Munck & Guyre, 1991). Corticosterone has also been implicated as the primary mediator of opioid-induced apoptosis of thymocytes resulting in thymic hypoplasia, a process reversible by adrenalectomy and mimicked by dexamethasone (Sei et al., 1991). Furthermore, dexamethasone has been shown to induce apoptosis in mature NK and cytotoxic T lymphocyte (CTL) cells *in vitro* and the addition of IL-2 or IL-4 protects against apoptosis (Migliorati et al., 1994).

The SNS is implicated in some forms of morphine-mediated immunosuppression through adrenergic pathways. Specifically, β-adrenoceptor antagonists have been shown to block morphine-mediated suppression of mitogen-induced lymphocyte proliferation (Fecho, Dykstra, & Lysle, 1993). α-Adrenergic antagonists (and to a lesser extent, β-adrenoceptor antagonists) have been shown to block suppression of splenic NK activity following acute morphine administration (Carr, Gebhardt, & Paul, 1993). Furthermore, recent data suggest that central rather than peripheral adrenergic pathways are involved in morphine-mediated suppression of splenic NK activity (Carr, Mayo, Gebhardt, & Porter, 1994a). These observations coincide with previous data showing the intracisternal administration of morphine resulted in the elevation of serum norepinephrine (NE), epinephrine (EPI), and dopamine from SNS stimulation of the adrenal medulla. The increase in monoamines was blocked by naloxone and the selective deinnervation of the adrenals (Van Loon, Appel, & Ho, 1981; Appel, Kirtsy-Roy, & Van Loon, 1986). Autonomic innervation of primary and secondary lymphoid tissue (Felten, Felten, Carlson, Olschowka, & Livnat, 1985) and the presence of α- and β-adrenergic receptors on lymphocytes have been demonstrated (McPherson, & Summers, 1982; Fuchs, Albright, & Albright, 1988), suggesting direct effects of catecholamines on cells of the immune system. Moreover, a decrease in the affinity and increase in the number of β-adrenergic receptors has been reported following acute morphine administration (Baddley, Paul, & Carr, 1993) supporting the notion of an intricate relationship between the adrenergic system and opioid-induced immunomodulation. Some immunomodulatory effects of morphine have also been found to be specific to the splenic but not to mesenteric lymph node lymphocytes, suggesting a high degree of compartmentalization consistent with SNS innerva-

tion of lymphoid tissue (Baddley et al., 1993; Lysle, Coussons, Watts, Bennett, & Dykstra, 1993).

Finally, the presence of opioid binding sites on lymphoid cells suggests a direct pathway of immunomodulation. Receptors for opioids are known to exist on lymphocytes (Madden, Donahoe, Zwemer-Collins, Shafer, & Falek, 1987; Carr, DeCosta, Kim, Jacobson, Guarcello, Rice, & Blalock, 1989; Ovadia, Nitsan, & Abramsky, 1989). These receptors have functional importance in immune homeostasis as indicated by *in vitro* measurement of immunocompetence (for review Carr, 1991).

The focus of this study was to assess the effect of chronic morphine exposure *in vivo* on the effector mechanisms of cell-mediated immunity, NK and CTLs measured *in vitro*. An 11-day chronic morphine treatment protocol was developed to investigate this question as well as address the significance of neuroendocrine influences on cell-mediated immunity. The intent of this initial study is to address long-term pain management, addiction, and withdrawal on immune homeostasis.

MATERIALS AND METHODS

Mice and Tumor Lines

Female C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) and C3H/HeN (Harlan-Sprague Dawley, Indianapolis, IN) mice were housed in groups of 6–10 per cage and maintained on a 12-h light/dark cycle. Access to water and food (Purina Mouse Chow) was available *ad libitum*. The YAC-1 mouse lymphoma cell line, P815 mastocytoma cell line, and EL-4 lymphoma cell line were obtained from the American Type Culture Collection (Rockville, MD); the cells have been maintained in culture by biweekly passage over the course of no more than 6 months/frozen lot.

Morphine Treatment Regimen

A dose–response study has established that 50.0 mg/kg of morphine sc results in maximal suppression of NK cytolytic activity (Carr, Gerak, & France, 1994b). In addition, preliminary results from dose effect studies indicates that 50.0 mg/kg elicits maximum suppression of CTL activity in alloimmunized mice (Carr, unpublished observation). This dose was used in all experiments.

In the chronic morphine exposure protocol, C3H/HeN mice (n = 23/group) were administered vehicle or morphine 2 h prior to receiving 1×10^7 C57BL/6J spleen cells, intraperitoneally (ip). Following the immunization, mice received vehicle or morphine daily for an additional 6 days. On Day 7, mice were reimmunized with 1×10^7 C57BL/6J spleen cells ip 2 h after the administration of vehicle or morphine. Following the second immunization, mice received morphine or vehicle daily for an additional 3 days. On Day 11, the mice were sacrificed and splenic lymphocytes (SL) were recovered and assayed for CTL and NK activity or processed further for other assays described below.

Lymphocyte Preparation

All mice were sacrificed by CO_2 asphyxiation and peritoneal lavage was performed using 10 ml of sterile Hank's buffer saline solution (HBSS). Cells were collected by recovering 10 ml of peritoneal fluid through a 20-gauge needle and 10-ml syringe. Spleens were removed and cell suspensions were prepared by

mechanical dispersion. SL and peritoneal exudate leukocytes (PEL) were washed with HBSS (250*g*, 5 min). Red blood cells were osmotically lysed using 0.84% NH_4CL; the cells were subsequently washed with HBSS (250*g*, 5 min) and resuspended in RPMI-1640 containing 10% fetal calf serum (FCS) and 2.5% Hybrimax (Sigma Chemical Co., St. Louis, MO) antibiotic (100 units/ml penicillin; 200 μg/ml streptomycin)/antimycotic (250 ng/ml amphotericin B) solution (complete medium). Cells were counted and examined for viability via trypan blue exclusion dye.

^{51}Cr-Release Cytolytic Assay

CTL activity was assayed using a 4-h microcytotoxicity assay with ^{51}Cr-labeled EL-4 (H-2^b) cells as targets. Between 5×10^4 and 160×10^4 effector cells were mixed with 1×10^4 target cells in conical 96-well microtiter plates (Costar, Cambridge, MA) in a reaction volume of 0.2 ml of complete medium. The cultures were incubated 4 h at 37°C in a 5% CO_2 atmosphere. A 100-μl aliquot of cell-free supernate was taken from each well and its ^{51}Cr content was determined using a Beckman gamma counter. The cytolytic activity was determined as follows: percentage cytolytic activity = [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(total cell-associated ^{51}Cr release − spontaneous ^{51}Cr release)] × 100 where "spontaneous" refers to ^{51}Cr release by target cells in the absence of effector cells. Total cell-associated ^{51}Cr was determined by measuring the ^{51}Cr content in the supernates of 10^4 target cells incubated at 37°C in a 5% CO_2 atmosphere in the presence of 0.1% *t*-octylphenoxypolyethoxyethanol (Triton X-100, Sigma Chemical Co.) in complete medium or measuring the ^{51}Cr content in 10^4 ^{51}Cr-labeled target cells. Spontaneous release was consistently between 10 and 15%. Each effector to target cell ratio was measured in triplicate/animal. One lytic unit (LU) was defined as the number of effector cells required to lyse 20% of the target cells per 10^7 total SL or PEL population. To determine antigen specificity for the CTL assay, P815 (H-2^d) mastocytoma cells were ^{51}Cr-labeled and used as third-party targets in the 4-h microcytotoxicity assay.

CTL Serine Esterase Assay

One hundred microliters of SL at a concentration of 2×10^6 cells/ml from C3H/HeN mice were placed in duplicate into 96-well conical-bottom microtiter plates. 10^5 irradiated (900 rads) C57BL/6J spleen cells in 100 μl of complete media were added to one set of wells. One hundred microliters 0.2% Triton X-100 in complete media was added to the other set as a measure of total activity. Cells were incubated for 2 or 4 h and 10 μl of supernate was removed from each well and transferred to the wells of a separate 96-well flat-bottom plate (Costar). To each well 190 μl of substrate consisting of 0.2 m*M* *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl ester (Sigma Chemical Co., St. Louis, MO) was added. Duplicate wells of complete media and 0.2% Triton X-100 were included as reagent blanks. The plates were incubated for 1 h and the optical density at 405 nm determined in a Dynatech MR5000 automatic plate reader. The esterase activity was determined as follows: percentage esterase activity = [(experimental absorbance − complete media blank absorbance)/(total absorbance-0.2% Triton X-100 absorbance)] × 100. Each well was read in triplicate.

$CD8^+$ Lymphocyte Enrichment

Mouse T cell subset enrichment columns (R & D Systems, Minneapolis, MN) were prepared as suggested by the manufacturer. SL from the groups were pooled as were SL from the morphine treatment groups and separately applied to CD8 enrichment columns. Recovered T cells were then assayed for CTL activity using ^{51}Cr-labeled EL-4 cells as targets in the ^{51}Cr-release cytolytic assay. Unfractionated splenic lymphocytes consisted of 12 ± 3% $CD8^+$ lymphocytes, whereas the $CD8^+$-enriched SL population consisted of 64 ± 5% $CD8^+$ lymphocytes, 0.0% $CD4^+$ lymphocytes, and 32 ± 6% B lymphocytes, macrophages, and null cells as determined by flow cytometry using a Coulter Elite (data not shown).

Serum Corticosterone and Dehydroepiandrosterone (DHEA) Assay

Animals were sacrificed by CO_2 asphyxiation and the thoracic cavity immediately opened and blood extracted via heart puncture with a 1-ml syringe and 27½-gauge needle. Samples were placed on ice overnight and exposed to the air to favor clot formation. Tubes were then spun at 10,000*g* for 1 min in a microcentrifuge (IEC; Needham, MA). Sera were removed and frozen at −20°C for later assay.

Sera from sacrificed animals were assayed for corticosterone by RIA using a corticosterone kit specific for rat and mouse corticosterone (ICN Biomedicals; Costa Mesa, CA) or DHEA (Diagnostic Products Corp., Los Angeles, CA). All samples were assayed simultaneously in duplicate. Standards were run simultaneously with experimental samples. The concentration of serum corticosterone and DHEA in the experimental samples was extrapolated from the curve generated from the known standards. The standard curve typically had a corresponding coefficient for linearity >.9900.

Cellular Cyclic Adenosine Monophosphate (cAMP) Assay

Enriched $CD8^+$ lymphocytes (10^6) in 200 μl of complete media were placed into two Falcon 2054 (Becton Dickinson; Lincoln Park, NJ) sterile snap cap tubes. To one of these tubes, 200 μl of complete medium was added (effector only, unstimulated); to the other tube was added 10^4 irradiated (900 rads) EL-4 target cells (effector + target, stimulated) in 200 μl of complete medium. A further tube containing 10^4 irradiated target cells in 400 μl of complete medium (target associated cAMP) was also prepared. All tubes were incubated at 37°C/5% CO_2 for 30 min. Parallel experiments were carried out representing vehicle and chronic morphine treatment groups. Following incubation, cells were immediately assayed for cAMP by enzyme-linked immunoabsorbent assay (ELISA) (ELISA Technologies; Lexington, KY). Standards were run simultaneously with experimental samples. The concentration of cAMP in the experimental samples was extrapolated from the curve generated from the known standards. The standard curve typically had a correlation coefficient for linearity >.9900.

Conjugate Formation Fluorescence-Activated Cell Sorter (FACS) Analysis

The protocol for labeling and measuring conjugates was carried out as described (Lebow & Bonavida, 1990) with modifications. Specifically, 5×10^5 $CD8^+$ lymphocytes from each treatment group (vehicle and chronic morphine) were placed into Falcon 2054 (Becton Dickinson; Lincoln Park, NJ) sterile snap cap tubes. An

additional tube of 5×10^5 enriched cells from vehicle-treated animals was also prepared (effector only). Cells were washed once with 1 ml PBS at 250*g* for 5 min, room temperature. Fluorescein isothiocyanate (FITC) dye was prepared by dissolving powdered FITC (Sigma, St. Louis, MO) in dimethyl sulfoxide (DMSO) at a concentration of 50 m*M* and diluting 1:1000 in phosphate-buffered saline (PBS, pH 7.4). Dye solution (100 μl) was added to the decanted washed cells and the cells then vortexed momentarily before being incubated at 37°C/5% CO_2 for 10 min with the caps dislodged. Cells were then washed twice as before with PBS and decanted. A volume of 250 μl of target cells (EL-4) at a concentration of 4×10^6 cells per milliliter of complete media was added to the cells from the experimental groups establishing an effector to target ratio of 1:2, favoring formation of single effector–target conjugates. Complete medium (250 μl) alone was placed into the tube containing effector cells alone and an additional tube containing 250 μl of the target cell suspension was prepared (target alone). Tubes were incubated for 90 min at 37°C/5% CO_2 with the caps dislodged and occasionally agitated gently to maintain suspension. Following the incubation, the cells were immediately placed on ice for FACS analysis. Immediately prior to FACS analysis, 5 μl of 0.1 μg/μl solution in phosphate-buffered saline (PBS, pH 7.4) of propidium iodide (Sigma) was added and the sample gently agitated to distribute the dye and ensure a uniform cell suspension.

FACS analysis was carried out on a Coulter Elite FACS (Coulter, Hialeah, FL). A log forward scatter vs log side scatter plot was used to gate viable cells for analysis, thereby separating whole cells from cell fragments. Whole effector only cells were plotted log green (FITC) vs log yellow (propidium iodide) which allowed effectors to be sorted for viability, needed for calculation of percentage conjugation in subsequent analysis of experimental samples. A log green vs log side scatter plot of effector only was gated so that viable effector cells were counted. The target-only sample was then analyzed with the above gates to ensure that target alone was not counted in the effector population. The vehicle experimental sample was analyzed and another gate was created to encompass the bulk of conjugates. An analysis window, cell count vs log yellow, was gated to the conjugates to allow for the calculation of percentage killing within the conjugated population. Analysis of the chronic morphine-treated sample proceeded with the above gate settings.

Calculation of percentage conjugation: percentage conjugation = (No. of conjugates/[No. of viable effectors + No. of conjugates]) × 100. Calculation of percent killing: percentage killing = (No. of gated dead cells within conjugate population/total gated conjugates) × 100. Effector cells only yielded 5 ± 2% uptake of PI. This number was used to subtract out percentage killing in the conjugate gate. Target cells only showed less than 1% PI uptake and were therefore not included in the background subtraction.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

RNA extraction. SL (1×10^7) RNA was extracted by vortexing in 1 ml of UltraSpec (Biotex, Houston, TX) RNA extraction solution in 1.5-ml microcentrifuge tubes. Samples were placed on ice for 5 min after which 0.2 ml chloroform was added followed by 15 s of vortexing and another 5-min incubation on ice. Samples were vortexed and then centrifuged for 15 min at 12,000*g* and 4°C. After centrifugation, 80% of the upper aqueous phase was removed and placed into

another 1.5-ml microcentrifuge tube. One volume of ice-cold isopropanol was added to the aqueous phase and the mixture was vortexed for 10 s and placed on ice for 20 min. The samples were then centrifuged for 10 min at 12,000*g* and 4°C. Samples were vacuum decanted and washed twice with ice-cold 75% ethanol, centrifuging for 10 min at 7500*g* and 4°C after each wash. Samples were then dried under vacuum and resuspended in 75 μl of sterile water. Optical density readings (Beckman DU-50 spectrophotometer) at 260 and 280 nm of a 1-μl aliquot were taken to determine the concentration and purity of RNA.

Reverse transcription. RNA samples were diluted to a concentration of 1 μg/3 μl water, 3 μl of each were placed into 0.5 ml microcentrifuge tubes suitable for temperature cycling. A RT master mix was prepared with the following reagents (Perkin Elmer Cetus, Norwalk, CT.) at these corresponding final concentrations: $MgCl_2$, 5 m*M;* 10× PCR buffer, 1×; dGTP, 1 m*M;* dCTP, 1 m*M;* dATP, 1 m*M;* dTTP, 1 m*M;* RNase inhibitor, 1 U/μl; RT, 2.5 U/μl; random hexamer primers, 2.5 μ*M*. A 17-μl aliquot of the master mix was added to each of the 1-μg samples. Tubes were submitted to temperature cycling (MJ Research) consisting of 42°C for 15 min followed by 99°C for 5 min and 5°C for 5 min.

Polymerase chain reaction. A PCR master mix was prepared with the following reagents (Perkin Elmer Cetus) at these corresponding final concentrations: $MgCl_2$, 2 m*M;* 10× PCR buffer, 1×; *Taq* DNA polymerase, 2.5 U/μl; relevant upstream primer 0.15 μ*M;* relevant downstream primer, 0.15 μ*M*. An 80-μl aliquot of the master mix was added to each of the samples which had been reverse transcribed. Tubes were again submitted to temperature cycles of 95°C for 2 min followed by 35 cycles of 1 min at 95°C and 1 min at 65°C followed by 7 min at 60°C and storage at 4°C.

Primers

G3PDH

5′–GTC–ATG–AGC–CCT–TCC–ACG–ATG–C–3′ upstream
5′–GAA–TCT–ACT–GGC–GTC–TTC–ACC–3′ downstream

POMC

5′–GAG–ATG–AAC–AGC–CCC–TGA–CTG–AAA–AC–3′ upstream
5′–AAT–GAG–AAG–ACC–CCT–GCA–CCC–TCA–CTG–3′ downstream

Route and Dose of Morphine and Herpes Simplex Virus-I

Vehicle (sc) or morphine (50 mg/kg, sc) was administered to C3H/HeN mice. Two hours following the drug administration, the LD_{50} of the McKrae strain of herpes simplex virus (HSV)-I (3×10^5 PFU) in RPMI-1640 was administered in the footpad of the mice in a volume of 50 μl. Subsequent to the virus administration, mice received vehicle (sc) or morphine (50 mg/kg, sc) daily up to the time of death of the animal or until the end of the observation period (21 days).

Reagents

Morphine sulfate was provided by the Research Technology Branch of The National Institute on Drug Abuse (Rockville, MD). Drug was dissolved in DMSO and diluted with HBSS to a concentration containing 25% DMSO. A volume of 100 μl of this solution containing the drug at the appropriate concentration was delivered to each mouse. Vehicle consisted of 25% DMSO in HBSS.

Statistics

One-way ANOVA (Randomized Block Design) was used together with Scheffé or Tukey's post hoc multiple comparisons test to determine significance ($p < .05$) between vehicle- and drug-treated groups. In some experiments, Bonferonni's t test was used to determine significance ($p < 0.05$) between vehicle- and drug-treated groups. In addition, the nonparametric Wilcoxon-signed rank test was used to calculate significant differences between the treated groups of animals. This statistical package used the GBSTAT program (Dynamic Microsystems Inc., Silver Springs, MD).

RESULTS

Chronic Morphine Exposure Suppresses CTL Activity

Mice treated with morphine for 11 days exhibited significantly less SL CTL activity compared to vehicle-treated controls (Fig. 1). PEL CTL activity was also significantly lower in the chronic morphine-treated compared to vehicle-treated mice (Fig. 2). However, both vehicle- and chronic morphine-treated mice showed similar levels of splenic NK activity (Fig. 3). SL from chronic morphine- and vehicle-treated mice were also assayed for lysis of a third-party target. Neither population of PEL or SL showed any measurable cytolytic activity against ^{51}Cr-labeled P815 cells (data not shown). In addition, SL from unprimed (nonimmunized) mice had no measurable CTL activity to the ^{51}Cr-labeled EL-4 targets (data not shown). Consistent with another study (Carpenter & Carr, submitted for publication) splenic CD8$^+$-enriched effector cells taken from mice treated daily with morphine over 11 days showed significantly less CTL activity compared to vehicle-treated controls (Fig. 4).

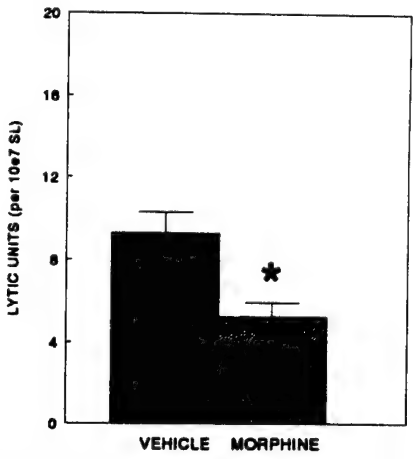

FIG. 1. Chronic morphine exposure suppresses splenic lymphocyte CTL activity. C3H/HeN mice (n = 23/group) were administered morphine (50.0 mg/kg, sc) or vehicle 2 h prior to alloimmunization (1×10^7 C57BL/6 SL, ip). Mice received morphine (50.0 mg/kg, sc) or vehicle daily for 9 days and were reimmunized 7 days after the primary immunization. Mice were sacrificed on Day 11 and the SL were assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. *$F(1,22) = 15.455$, $p < .05$ comparing vehicle- to chronic morphine-treated mice as determined by one-way ANOVA and Scheffe multiple comparison test. Bars represent SEM, $n = 23$.

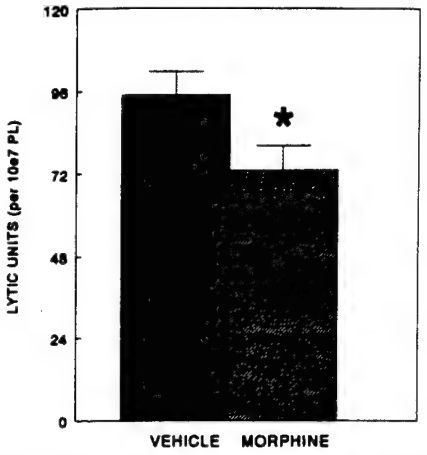

FIG. 2. Chronic morphine exposure suppresses CTL activity of peritoneal exudate leukocytes. Mice were treated as described in the legend of Fig. 1. PEL were collected and assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. *$F(1,22) = 6.3882$, $p < .05$ comparing vehicle- to chronic morphine-treated mice as determined by ANOVA followed by Scheffe multiple comparison test. Bars represent SEM, $n = 23$.

Serine Esterase Release Is Reduced in Response to Antigen in SL from Chronic Morphine-Treated Animals

Serine esterases are contained within the granules of CTLs and are released upon contact with target (Pasternack & Eisen, 1985; Pasternack, Verret, Liu, & Eisen, 1986; Young, Leong, Liu, Damiano, & Cohn, 1986). To further investigate the reduced cytolytic activity found in the chronic morphine-treated animals, serine esterase release and granulation was measured. Serine esterase release from SL in response to alloantigen stimulation yielded results which paralleled the SL CTL activity. Specifically, after 2 h exposure to irradiated targets (C57Bl/6J splenocytes) the percentage of serine esterase content in the supernates was significantly lower in the SL taken from chronic morphine-treated mice com-

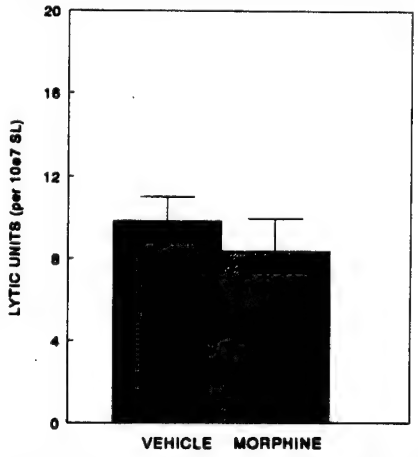

FIG. 3. Chronic morphine exposure has no effect on splenic NK activity. Mice were treated as described in the legend of Fig. 1. SL were assayed for NK activity using ^{51}Cr-labeled YAC-1 cells. Bars represent SEM, $n = 23$.

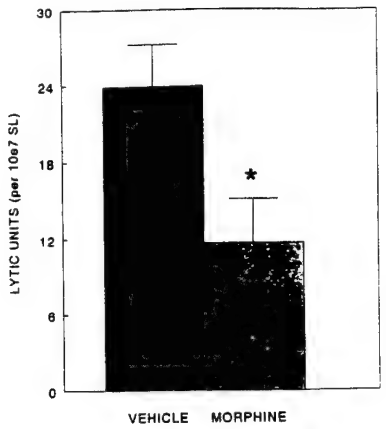

FIG. 4. Chronic morphine exposure suppresses $CD8^+$-enriched CTL activity. C3H/HeN mice (n = 4/group) were treated as described in the legend of Fig. 1. Mice were sacrificed on Day 11 and the SL were pooled within treatment groups, enriched for $CD8^+$ cells, and assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. *$F(1,5) = 6.5794$, $p < .05$ comparing vehicle- to chronic morphine-treated mice as determined by ANOVA and Scheffe multiple comparison test. Bars represent SEM, $n = 4$.

pared to serine esterase release from the vehicle-treated controls (Table 1). In addition, there was a significant decrease in the total serine esterase content of SL taken from chronic morphine compared to vehicle-treated mice at the 2-h time point (Table 1). By 4 h postincubation, the differences were no longer significant (Table 1).

Cyclic AMP Levels of Enriched $CD8^+$ Cells from Chronic Morphine-Treated Animals Revealed a Decreased Response to Antigen

Cyclic AMP has been implicated as the second messenger responsible for the termination of attack between effector and target (Valitutti, Dessing, & Lanza-

TABLE 1
Morphine Suppresses the Release and Total Cell-Associated Serine Esterase (SE) Level in CTL Cells[a]

	SE content in supernatant[b]		Cell-associated SE[c]	
Treatment	2-h	4-h	2-h	4-h
Vehicle	13.3 ± 2.2	25.5 ± 4.7	.097 ± .008	.046 ± .007
Morphine	8.4 ± 1.9*	18.4 ± 3.7	.066 ± .002*	.039 ± .004

[a] Splenic lymphocytes from immunized mice were assayed for SE content following reexposure to antigen.

[b] Cell-free supernatant from mixed lymphocyte reactions were collected and assayed for SE at the designated time points. The numbers represent the percentage of the total cell-associated SE. Splenic lymphocytes from unprimed C3H/HeN mice were used to determine baseline SE levels. The baseline levels were subtracted from the experimental percentages.

[c] Total cell-associated SE was determined following lysis (0.1% Triton X-100 in complete medium) of the C3H/HeN splenic lymphocytes. Numbers are in absorbance read at 405 nm (background subtracted).

* $F(1,11) = 6.3307$, $p < .05$ for the percentage SE content in supernatant; $F(1,4) = 9.9415$, $p < .05$ for cell-associated SE comparing morphine-treated to vehicle-treated controls as determined by one-way ANOVA and Scheffe multiple comparison test.

recchia, 1993). A rise in intracellular cAMP has been associated with detachment from the target cell and a halt in degranulation ("preservation of granulation") (Valitutti et al., 1993). Cyclic AMP has also been implicated as the signal responsible for the initiation of recycling of CTL for subsequent lytic function (Valitutti et al., 1993).

To further examine mechanisms involved in the suppression seen in the chronic morphine-treated animals, measurements of intracellular cAMP in enriched $CD8^+$ cells were made in response to antigen stimulation *in vitro*. Results indicate that the increase in cAMP of $CD8^+$ cells derived from chronic morphine-treated animals is reduced in response to target cells when compared to the response in the $CD8^+$-enriched effector cells from vehicle-treated animals (Table 2).

Ability of $CD8^+$ Cells Derived from Chronic Morphine-Treated Mice to Form Conjugates Is Not Significantly Impaired

In an attempt to correlate conjugate formation with reduced cytolytic activity, a study of the ability of the cells to form conjugates and kill targets was undertaken. The results revealed no significant differences in conjugate formation in the cells derived from chronic morphine-treated versus vehicle-treated animals following a 90-min incubation period. Of the gated events (viable effector cells conjugated to targets), 45.1 ± 1.8% were conjugated to target cells of the enriched $CD8^+$ effector cells from vehicle-treated mice compared to 40.7 ± 5.5% of the effector cells from the chronic morphine-treated group. Of the cells engaged in conjugation, 3.4 ± 1.1% of the conjugated targets were dead in the vehicle group compared to 3.1 ± 0.7% in the morphine group. Consistent with these results, cell surface expression of the CD11a adhesion molecule necessary for the formation of effector–target conjugates was similar in both $CD8^+$ SL from vehicle- and chronic morphine-treated animals (Table 3).

TABLE 2
Chronic Morphine Exposure Attenuates cAMP Production Following Antigen Stimulation in $CD8^+$ Effector Cells

Experiment	Treatment	Effector only	Effector + target
1	Vehicle	8.9 ± 1.8[a]	13.2 ± 0.8
	Morphine	10.3 ± 1.7	10.7 ± 2.1
2	Vehicle	9.1 ± 0.1	11.0 ± 0.7
	Morphine	5.0 ± 1.9	5.0 ± 2.0
3	Vehicle	10.1 ± 0.7	16.9 ± 2.8
	Morphine	9.0 ± 0.7	14.1 ± 3.7
4	Vehicle	6.2 ± 0.9	11.6 ± 0.5
	Morphine	5.6 ± 0.8	6.5 ± 0.6
Summary	Vehicle	8.6 ± 0.8	13.2 ± 1.3*
	Morphine	7.5 ± 1.3	9.1 ± 2.1

[a] Numbers are expressed in pmols/10^6 effector cells ± SEM, $n = 4$. Target cells alone yielded 1.1 ± 0.5 pmol/10^4 cells.

* $F(3,15) = 2.9738$, $p < .05$ comparing effector only to effector + target in the summary vehicle group as determined by one-way ANOVA and Tukey's post hoc t test.

TABLE 3
Expression of CD11a on $CD8^+$ Lymphocytes Is Not Altered after Chronic Morphine Exposure

Treatment	Percentage $CD11a^{+a}$	Percentage $CD11^+CD8^+$
Vehicle	75.9 ± 0.6	10.5 ± 0.6
Morphine	81.8 ± 0.9*	12.5 ± 0.6

[a] Total percentage of $CD11a^+$ expressing splenic lymphocytes ± SEM, $n = 6$ vehicle-treated, $n = 5$ morphine-treated.

* $p < .05$ comparing morphine-treated to vehicle-treated mice as determined by Bonferonni's t test.

Serum Corticosterone Levels Are Significantly Lower after Chronic Treatment with Morphine

To investigate the role of corticosterone in the suppression of cytolytic activity resulting from chronic morphine exposure, sera were assayed for corticosterone in parallel with cytolytic assays of $CD8^+$-enriched cells. Serum corticosterone levels in vehicle-treated animals were significantly elevated compared to those of morphine-treated mice (Fig. 5A). Likewise, DHEA levels were decreased in the chronic morphine-treated mice compared to the vehicle-treated controls (Fig. 5B).

Expression of POMC in SL Does Not Correlate with Chronic Morphine- or Vehicle-Treated Animals

Two of the important products of the POMC gene are adrenocorticotropic hormone (ACTH) and opioid peptides (e.g., α, β, and γ endorphins). An alternative source of ACTH may be lymphocytes (Smith, Meyer, & Blalock, 1982). To investigate these cells as a source of immunomodulation, the expression of the POMC gene in the SL was undertaken. SL RNA from individual animals within the chronic morphine and vehicle experimental groups was submitted to RT.

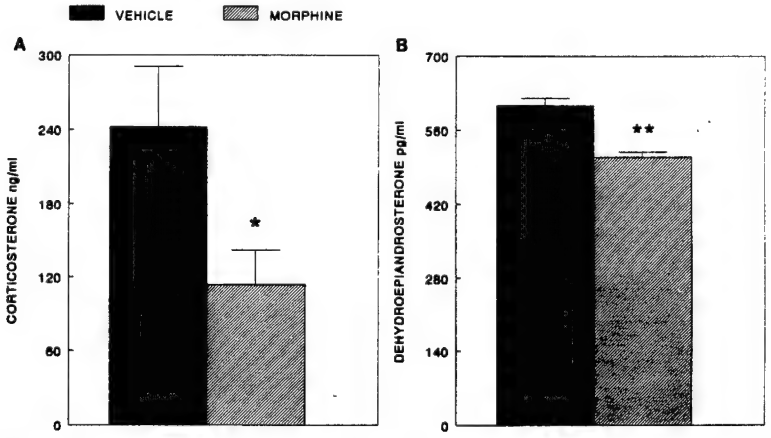

FIG. 5. Chronic morphine exposure reduces serum corticosterone and serum DHEA levels. C3H/HeN mice were treated as described in the legend of Fig. 1. Upon sacrifice, blood was obtained by cardiac puncture and assayed for corticosterone and DHEA level by radioimmunoassay. (A) Analysis of serum corticosterone levels. *$p < .05$ comparing vehicle- to chronic morphine-treated mice as determined by Bonferroni's t test. Bars represent SEM, $n = 11$ (vehicle) or 13 (chronic morphine) animals tested. (B) Analysis of serum DHEA levels. **$F(1,29) = 32.0516$, $p < .01$ comparing vehicle- to chronic morphine-treated mice as determined by ANOVA and Scheffe multiple comparison test. Bars represent SEM, $n = 15$/group.

Products of reverse transcription were then amplified by PCR using primers specific for G3PDH and Exon 3 of POMC. Positive control RNA (from AtT-20 corticotroph cell line) was reverse transcribed and amplified in parallel with experimental samples. No pattern could be discerned comparing the drug- to vehicle-treated groups (Fig. 6). Specifically, lymphocytes from 3 of 13 vehicle animals were positive for exon 3 POMC-amplified product, while the lymphocytes of 6 of 14 chronic morphine-treated animals screened were positive. All animals had equivalent levels of G3PDH amplified product (Fig. 6).

Chronic Morphine Exposure Reduces the Survival Rate of C3H/HeN Mice Infected with Herpes Simplex Virus Type I(HSV-1)

To further define the biological significance of chronic morphine exposure, C3H/HeN mice were infected with an LD_{50} of the McKrae strain of HSV-1. One of 12 chronic morphine-treated mice infected with the virus survived the 21-day observation period (Fig. 7). Moreover, 9 of 12 morphine-exposed mice had succumbed to the infection within 7 days of virus administration. In comparison, 3 of 12 vehicle-treated mice survived the virus infection with 6 of 12 mice succumbing to infection 7 days following virus administration.

DISCUSSION

In the present study, we have investigated the immunomodulatory effect of chronic morphine exposure on NK and CTL activity. Short-term (daily exposure to morphine for 5 days) morphine (50.0 mg/kg, sc) administration did not modify CTL activity in alloimmunized C3H/HeN mice (Carr, unpublished observation). In addition, the generation of CTLs in *in vitro* one-way mixed lymphocyte reactions is unaffected in the presence of morphine (10^{-5}–10^{-11} *M*) (Carr & Carpenter, submitted), suggesting that morphine does not directly act on immune cells in the context of CTL generation. However, chronic morphine exposure does sup-

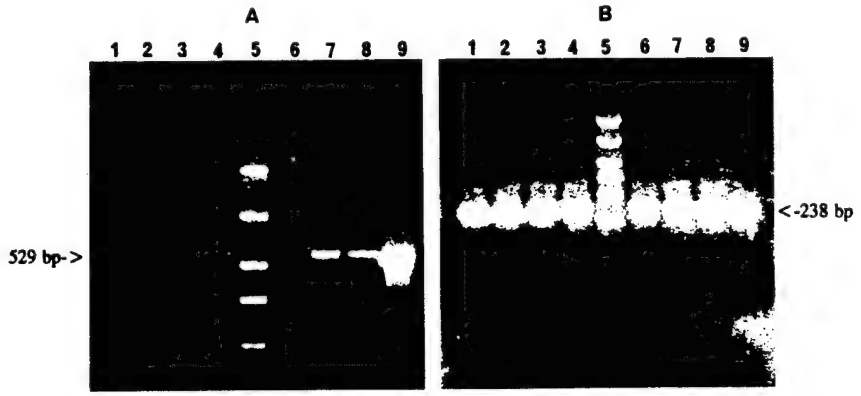

FIG. 6. POMC transcript levels do not coincide with morphine-mediated suppression of splenic CTL activity. SL RNA was subjected to RT-PCR using oligonucleotide probes specific for exon 3 of POMC or G3PDH as described under Materials and Methods section. Lanes 1–4, RNA from SL from vehicle-treated mice; lane 5, DNA ladder in descending order: 1000, 700, 500, 400, 300, and 200 bp; lanes 6–8, RNA from SL from chronic morphine-treated mice; lane 9, RNA from AtT-20 pituitary tumor cells. (A) RT-PCR amplification using POMC primers resulting in a 529-bp product. (B) RT-PCR amplification using G3PDH primers resulting in a 238-bp product.

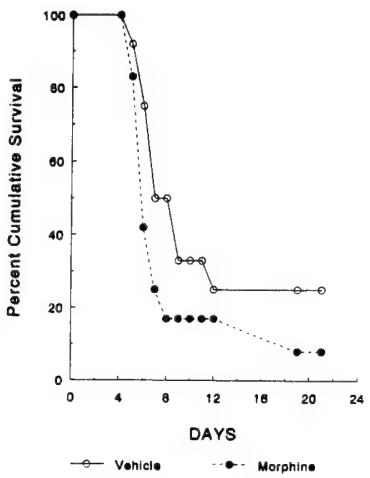

FIG. 7. The effect of chronic morphine treatment on the percentage of cumulative survival of C3H/HeN mice following HSV-I infection. Mice were injected into the footpad with the LD_{50} of the McKrae strain of HSV-I 2 h after receiving vehicle or morphine as described (see Materials and Methods). $p < .001$ comparing morphine-treated to vehicle-treated mice percentage survival as determined by the nonparametric Wilcoxon signed rank test, $z = 8.29$.

press CTL activity in alloimmunized mice and this effect is blocked by β-funaltrexamine (μ-selective opioid receptor antagonist) (Carpenter & Carr, submitted for publication) but not (*E*)-7-benzylidine-7-dihydronaltrexone (δ-selective opioid receptor antagonist) pretreatment (Carr & Carpenter, submitted for publication). Moreover, the suppression in CTL activity does appear to be modestly significant since only 8% of HSV-I-infected mice chronically treated with morphine survived the infection, while 25% of vehicle-treated HSV-I-infected mice survived. The reduction in percentage survival of vehicle-treated mice following the LD_{50} for this particular strain of HSV-I may be due to the daily handling and injections, resulting in a short-term "stressed state."

Compartmentalization of morphine-induced effects on immune functions is cited in some studies as evidence for the involvement of the SNS (Baddley et al., 1993; Lysle et al., 1993). This appears to be justified given the direct innervation of lymphoid tissue by fibers of the SNS (Felten, Felten, Bellinger, Carlson, Ackerman, Madden, Olschowka, & Livnat, 1987). The SNS uses norepinephrine almost exclusively as a mediator. The SNS, however, is not the sole source of norepinephrine. *In vivo,* the adrenal medulla produces epinephrine, dopamine, and norepinephrine and is controlled via the SNS as well. In addition, opioids are capable of activating this pathway (Van Loon et al., 1981; Appel et al., 1986). Accordingly, the global immunomodulatory effects of morphine could be mediated by SNS stimulation of the adrenal medulla, resulting in the release of sufficient quantities of catecholamines to exert a systemic immunomodulatory effect.

In vitro catecholamines have been shown to have immunomodulatory effects on indicators of CTL function. The lytic activity of CTL was found to be potentiated by the addition of NE, EPI, or isoproterenol (β-agonist) at the beginning of culture, coaddition of the β-blocker timolol abolished the augmentation (Felten et al., 1987; Livnat, Madden, Felten, & Felten, 1987). *In vivo,* chemical sympathectomy has been shown to reduce CTL activity and influence lymphocyte trafficking

(Madden & Livnat, 1991). Combined, the action of catecholamines suggest that their effect is dependent on concentration, timing, cell type, and site of action. For example, early effects on CTL activation and differentiation appear to potentiate activity, whereas late effects inhibit CTL effector function (Strom & Carpenter, 1980).

The effect of endogenous opioids on CTL generation *in vivo* has not been revealed. Unlike morphine, endogenous opioid peptides have been found to potentiate the generation of CTLs *in vitro* through a naloxone-sensitive mechanism (Carr & Klimpel, 1986). To investigate a possible role for these peptides (i.e., endorphines) *in vivo,* we examined the expression of the POMC gene by SL harvested from chronic morphine- and vehicle-treated animals. Results of RT-PCR amplifications of SL RNA failed to reveal a correlation between the expression of exon 3 POMC transcripts and the treatment group. This suggests that the POMC gene may be expressed only transiently in SL and is not an important component in the suppression of cytolytic activity found in the effector population.

The result showing a reduced corticosterone level in the chronic morphine-treated animals relative to vehicle-treated mice is unexpected. Previous studies have shown HPA axis involvement in modifying the immune system following short-term exposure to morphine (Bryant et al., 1991; Sei et al., 1991; Fuchs & Pruett, 1993). However, the present results would indicate the levels of corticosterone do not coincide with suppression of CTL activity. Moreover, recent results have shown short-term exposure to morphine (50 mg/kg daily for 5 days) *in vivo* has no effect on the generation of CTLs in alloimmunized C3H/HeN mice (Carr, unpublished observation), suggesting the influence of corticosterone in CTL generation or activity is minimal in this strain of mouse. However, in CBAxC57BL/6 mice, short-term exposure to morphine (50 mg/kg daily for 5 days) *in vivo* modifies CTL activity (Garza, Prakash, & Carr, submitted for publication), indicating the strain-specific nature of morphine-mediated immunomodulation as previously reported (Bussiere et al., 1992) as well as the potential role of the HPA axis.

In the present study, circulating levels of the adrenal cortical steroid hormone DHEA were found to be modestly but significantly lower in the morphine-treated mice. DHEA has previously been shown to protect mice against a lethal dose of virus (coxsackievirus and herpes simplex virus type II) (Loria, Inge, Cook, Szakal, & Regelson, 1988) and is predicted to interfere with the immunosuppressive effects of corticosterone (Riley, 1983). Recently, androstenediol, a metabolic product of DHEA has been found to be 100× more potent than DHEA in regulating resistance to viral and bacterial infections (Loria & Padgett, 1992). In terms of the present study, the results suggest that the increase in HSV-I-elicited encephalitis and death in chronic morphine-treated mice may be due in part to a reduction in the circulating levels of DHEA and metabolites, thus eliminating the endogenous corticosterone antagonist. However, the observation showing that corticosterone levels were also significantly lower in the chronic morphine-treated mice seems to suggest that the adrenal glands may not have been functioning correctly.

Early events in CTL target cell recognition include target cell adhesion followed by "programming" for lysis. Within 2–10 min following target cell adhesion cytoplasmic granules within the CTL reorients to the region near the interface with the target cell (Englehard, Gnarra, Sullivan, Mandell, & Gray, 1988). This pro-

gramming for lysis results from the activation of pathways involving phospholipase C (PLC), phosphatydylinositol turnover, and protein kinase C. A sharp increase in intracellular calcium from intracellular and extracellular sources promotes reorientation, fusion, and exocytosis of granules (Englehard et al., 1988; Ostergaard & Clark, 1987). Granule fusion is terminated simultaneously with a sharp increase in intracellular cAMP (Valitutti et al., 1993).

In an effort to identify the mechanism(s) for suppressed cytolytic activity, we investigated the ability of purified CTLs from chronic morphine- and vehicle-treated mice to form conjugates and subsequently lyse targets. A previous study showed that acute morphine administration suppressed conjugate formation and cytolysis of target cells by NK-enriched effector cells (Carr et al., 1994a). The results in the present investigation indicate that the ability of CTLs from chronic morphine-treated animals to form conjugates with allogeneic target cells at the 90-min time point is not impaired. This is consistent with the finding that there were no significant differences in CD11a expression by purified effectors cells between the vehicle- and chronic morphine-treated groups. Differences in CD11a expression would presumably result in differences in the avidity between effector–target conjugates resulting from the specific interactions between CD11a on effector cells and CD54 (ICAM-1) on the target cells (Dustin & Springer, 1989; Nakamura, Takahashi, Fukazawa, Koyanagi, Yokoyama, Kato, Yagita, & Okumura, 1990; Spits, Schooten, Keizer, Seventer, Rijn, Terhorst, & Vries, 1986). Subsequent killing of targets was likewise not impaired. This result seems contrary to the deficient killing observed in the cytolytic assays. However, the original cytolytic assays were carried out over 4 h, presumably allowing for multiple effector–target interactions with effectors recycling after initial attack to kill again (Valitutti et al., 1993). The conjugate studies, on the other hand, span 1.5 h, which perhaps is insufficient time *in vitro* for attack and recycling. Measurements made at 2.5–3 h were inconclusive due to the high background associated with propidium iodide uptake by effector and target cells.

The two groups of enriched CTL, chronic morphine-treated vs vehicle-treated, are not equivalent. One major difference is the granulation found in SL from the chronic morphine-treated animals. These results suggest that production of esterase-containing granules by the CTL subpopulation is deficient. Assuming that at the time of harvest enriched $CD8^+$ CTL from the chronic morphine- and vehicle-treated animals have both cleared the antigen stimulus, it is probable that the CTL population has recycled and awaits new targets. If at the time of harvest these CTLs represent a population "awaiting" new target then it is logical that the level of CTL granulation present represents a maximum constitutive level in the chronic morphine- and vehicle-treated groups. The 1.5-h conjugate studies of purified CTL show equivalent ability to form conjugates combined with equivalent capacity to deliver a lethal hit, but killing is impaired in the chronic morphine-treated group in the 4-h cytolytic assay results. The conjugate results, however, represent only a primary contact with target, which suggests that the level of granulation present in the awaiting CTL from chronic morphine- and vehicle-treated animals are both sufficient to deliver an initial lethal hit although their respective initial level of granulation is different. The subsequent secondary and tertiary contact with the target cells is likely to be where the defect resides. Accordingly, this points to a possible defect in CTL recycling in the chronic morphine-treated animals. The results of the cAMP studies suggest a mechanism

for this result. An increase in intracellular cAMP is associated with termination of attack after a positive interaction with target and believed to be the secondary signal that induces detachment from target, initiation of recycling, and preservation of granulation (Valitutti et al., 1993). The cAMP levels in CTLs from chronic morphine-treated animals after 30 min of antigen exposure appears reduced although basal levels in unstimulated cells are similar to that of the vehicle-treated animals. This suggests that the CTL derived from the chronic morphine-treated animals are defective in the termination of attack, and this may prolong CTL contact with target and lead to excessive degranulation and delayed recycling. Together these processes might impair subsequent killing or simply reduce the total number of lytic contacts in the 4-h time period of the cytolytic assays. In summary, two pathways of chronic morphine treatment are proposed: (1) Esterase content of CTLs is reduced. (2) Termination of attack is impaired, leading to impaired recycling and excessive degranulation and/or prolonged contact leading to a reduction in total contacts within the time frame of the assays.

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Immunopharmacology 29 (1995) 129–140

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Pretreatment with β-funaltrexamine blocks morphine-mediated suppression of CTL activity in alloimmunized mice

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Abstract

The effect of prolonged exposure to morphine on cytotoxic T lymphocytes (CTL) and splenic natural killer (NK) activity was investigated. Daily administration of morphine (50.0 mg/kg, s.c.) to alloimmunized mice for 11 days resulted in a significant decrease (25–50%) in peritoneal and splenic CTL activity but not splenic NK activity. To identify the effector cell population mediating cytolysis, cell enrichment studies were carried out. The results of these studies indicated the CTLs are $CD8^+$ $CD4^-$. Chronic morphine treatment increased the percentage (25–30%) of $CD3^+CD4^+$ and $CD8^+$, but not Ig^+ cells in the spleen relative to saline-treated controls. Pretreatment of mice with the μ-selective antagonist, β-funaltrexamine blocked morphine-mediated suppression of splenic and peritoneal CTL activity as well as the increase in $CD3^+CD4^+$ and $CD8^+$ splenic lymphocytes. These results indicate the generation of CTLs in vivo is sensitive to chronic morphine exposure implicating opiates as important co-factors through modulation of cell-mediated immunity.

Keywords: Cytotoxic T lymphocyte; Morphine; Natural killer activity; β-Funaltrexamine

1. Introduction

The abuse of opioid compounds is predicted to be a major co-factor in the acquisition and spread of human immunodeficiency virus (HIV)-1 (Donahoe, 1992) due to the immunosuppressive side-effects of such drugs. Specifically, morphine has been shown to suppress picryl chloride-induced delayed-type hypersensitivity (Bryant and Roudebush, 1990), splenic NK activity (Shavit et al., 1984; Weber and Pert, 1989), primary antibody production (Pruett et al.,

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Abbreviations: BSA, bovine serum albumin; CTL, cytotoxic T lymphocyte; DMSO, dimethylsulfoxide; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; β-FNA, β-funaltrexamine; HBSS, Hanks' balanced salt solution; HIV, human immunodeficiency virus; HPA, hypothalamic-pituitary adrenal; Ig, immunoglobulin; MLC, mixed lymphocyte culture; NK, natural killer; PBS, phosphate-buffered saline; PE, phycoerythrin; PL, peritoneal exudate leukocyte(s); PWM, pokeweed mitogen; SL, splenic lymphocyte(s); TdR, thymidine deoxyribonucleic acid.

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1992; Bussiere et al., 1993) and resistance to viral infections (Lorenzo et al., 1987; Starec et al., 1991). Similarly, immunocompetence is compromised in human heroin users (Novick et al., 1989; DeShavo et al., 1989; Klimas et al., 1991) resulting in greater susceptibility to infectious agents (Dismukes et al., 1968) including HIV-1 (Hubbard et al., 1988). The acute administration of morphine suppresses NK activity through the activation of α-adrenergic pathways (Carr et al., 1993; 1994a) while chronic morphine administration activates the hypothalamic-pituitary adrenal (HPA) axis resulting in elevated levels of corticosteroids which are, in part, immunosuppressive (Bryant et al., 1991). However, the relationship between the HPA axis and other neuroendocrine pathways relative to chronic opioid-induced modulation of immune homeostasis (specifically NK and CTL activity) is still largely unknown.

In the present study, the effects of chronic administration of morphine on CTL activity in mice was investigated. Previous studies have shown the endogenous opioid peptides [Met]-enkephalin and β-endorphin augment the generation of CTLs in one-way mixed lymphocyte cultures (MLCs) in vitro through a naloxone-sensitive pathway (Carr and Klimpel, 1986). Since opioid abusers show an increased susceptibility to viral infections (Dismukes et al., 1968; Hubbard et al., 1988), an investigation assessing the immunomodulatory characteristics of morphine on CTL activity was undertaken.

2. Materials and methods

2.1. Mice and tumor lines

Female C57BL/6J (The Jackson Laboratory, Bar Harbor, ME, USA) and C3H/HeN (Harlan-Sprague Dawley, Indianapolis, IN, USA) mice were housed in groups of 6–10 per cage and maintained on a 12-h light/dark cycle. Access to water and food (Purina Mouse Chow) was available ad libitum. The YAC-1 mouse lymphoma cell line, P815 mastocytoma cell line and EL-4 lymphoma cell line were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA); the cells have been maintained in culture by biweekly passage over a 5-month period.

2.2. Morphine treatment regimen

A dose-response study has established that 50.0 mg/kg of morphine s.c. results in maximal suppression of cytolytic activity (Carr et al., 1994b). Consequently, this dose was used in all experiments.

C3H/HeN mice ($n = 8$/group) were administered the μ-opioid receptor alkylating agent β-funaltrexamine (β-FNA, 40.0 mg/kg, s.c.) or vehicle 18–24 h prior to receiving morphine (50.0 mg/kg). 2 h following morphine or vehicle administration, mice received 1×10^7 C57BL/6 spleen cells, i.p. Following the immunization, mice received vehicle or morphine daily for an additional 6 days. On day 7, mice were re-immunized with 1×10^7 C57BL/6 spleen cells, i.p. 2 h after the administration of vehicle or morphine. In addition, mice received vehicle or β-FNA (40.0 mg/kg, s.c.) every 72 h (immediately following the subsequent morphine administration) up through the 10-day incubation period. This time period corresponds to opioid receptor turnover as a result of occupancy of receptors by β-FNA (D. Paul, personal communication). On day 11, the mice were sacrificed and splenic lymphocytes (SL) and peritoneal exudate leukocytes (PL) were collected and assayed for mitogen responsiveness, CTL and NK activity. The phenotypes of the spleen cells of vehicle- and drug-treated mice were determined by flow cytometry.

2.3. SL and PL preparation

All mice were killed by CO_2 asphyxiation and peritoneal lavage was performed using 10 ml of sterile Hanks' balanced salt solution (HBSS). Cells were collected by recovery of peritoneal lavage fluid through a 20 gauge needle and 10 ml syringe. Spleens were removed and cell suspensions were prepared by mechanical dispersion. SL and PL were washed with HBSS ($250 \times g$, 5 min). Red blood cells were osmotically lysed using 0.84% NH_4Cl; the cells were subsequently washed with HBSS ($250 \times g$, 5 min) and resuspended in RPMI-1640 containing 10% fetal calf serum (FCS) and 2.5% Hybri-max (Sigma, St. Louis, MO, USA) antibiotic/antimycotic solution (complete media). Cells were counted and examined for viability using Trypan blue exclusion.

2.4. Mitogen-induced proliferation assay

SL from C3H/HeN mice (2×10^5 cells/well) in 100 μl of complete media were placed in 96-well microtiter plates (Costar, Cambridge, MA, USA). 100 μl of complete media containing 100 ng pokeweed mitogen (PWM) was added to the wells. Spleen cells were cultured in 5% CO_2 at 37°C for 48 h. After 48 h, 200 nCi [^{3}H]thymidine deoxyribonucleic acid (TdR) in HBSS (10 μl) was added to each well and the cells cultured an additional 12 h. The cells then were harvested on glass fiber filter strips using a multiple-well harvester (Cambridge Technologies, Watertown, MA, USA). Filters were placed in scintillation vials containing 6.0 ml of Cytoscint liquid scintillation cocktail (ICN, Irvine, CA, USA) and allowed to equilibrate 18–24 h. The incorporation of [^{3}H]TdR was determined by liquid scintillation counting using a Beckman LS9800. The mitogenic response of spleen cells from each animal was assayed in quadruplicate. Incorporation of [^{3}H]TdR by cells cultured in the absence of PWM was less than 5% of that obtained in maximally stimulated cultures.

2.5. ^{51}Cr-release cytolytic assay

SL and PL CTL activity was assayed using a 4-h microcytotoxicity assay with ^{51}Cr-labeled EL-4 cells (H-2^b) as targets. Between 5×10^4 and 160×10^4 effector cells were mixed with 1×10^4 target cells in conical 96-well microtiter plates (Costar) in a reaction volume of 0.2 ml of complete media. The cultures were incubated 4 h at 37°C in a 5% CO_2 atmosphere. A 100-μl aliquot of cell-free supernatant was taken from each well and its ^{51}Cr content was determined using a Beckman gamma counter. The cytolytic activity was determined as follows: percent cytolytic activity = ((experimental ^{51}Cr release – spontaneous ^{51}Cr release)/(total cell-associated ^{51}Cr release – spontaneous ^{51}Cr release)) × 100 where 'spontaneous' refers to ^{51}Cr release by target cells in the absence of effector cells. Total cell-associated ^{51}Cr was determined by measuring the ^{51}Cr content in the supernates of 10^4 target cells incubated at 37°C in a 5% CO_2 atmosphere in the presence of 0.1% *t*-octylphenoxypolyethoxyethanol in complete medium or measuring the ^{51}Cr content in 10^4 ^{51}Cr-labeled target cells. Spontaneous release was consistently between 10–15%. Each effector to target cell ratio (100:1, 50:1, 25:1 and 12:1 for SL, and 50:1, 25:1, 12:1 and 6:1 for PL) was measured in triplicate/animal. One lytic unit (LU) is defined as the number of splenic lymphocytes, peritoneal lymphocytes or enriched effector cells able to lyse 20% of the target cells (YAC-1 or EL-4 target cells) and this unit is expressed per 10^7 total cells. To determine antigen specificity for the CTL assay, P815 (H-2^d) mastocytoma cells were ^{51}Cr-labeled and used as targets in the 4-h microcytotoxicity assay.

2.6. CD4$^+$ and CD8$^+$ lymphocyte enrichment

Mouse T-cell subset enrichment column kits (R&D Systems, Minneapolis, MN, USA) were prepared as suggested by the manufacturer. SL from the saline-treated group were pooled as were SL from the morphine treatment group and separately applied to CD4 and CD8 enrichment columns. Recovered T cells were then assayed for CTL activity using ^{51}Cr-labeled EL-4 cells as targets in the ^{51}Cr release cytolytic assay.

2.7. Fluorescence-activated cell sorter (FACS) analysis of SL subpopulations

SL (1×10^6 cells/condition) obtained from the vehicle- and drug-treated groups of mice were collected and washed in 1.0 ml of phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.05 M NaN_3. SL were resuspended in 0.05 ml PBS-BSA plus NaN_3 containing 30 μg of rat IgG_{2b} (isotypic control; Zymed, South San Francisco, CA, USA) and incubated on ice for 10 min. Subsequently, antibody to CD3, CD4 and/or CD8 (rat IgG_{2b}; Gibco BRL, Gaithersburg, MD, USA) conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or α-immunoglobulin (Ig, heavy- and light-chain-specific, F(ab)$_2$) conjugated with FITC (Boehringer-Mannheim, Indianapolis, IN, USA) was added for a final volume of 0.1 ml (using PBS-BSA plus NaN_3). The labeled cells were allowed to incubate for 30 min on ice in the dark. The cells were washed with ice-cold PBS, fixed with 1% paraformaldehyde and analyzed by

FACS for the percentage of stained cells in the cell population. Light scatter was collected at 488 nm and the emitted light which passed through a long pass filter was analyzed at 525 nm (FITC) or 575 nm (PE) on a Coulter Elite FACS (Coulter, Hialeah, FL, USA). 5000 gated events were analyzed per sample.

2.8. Reagents

Morphine sulfate and β-FNA were generously provided by the Research Technology Branch of The National Institute on Drug Abuse (Rockville, MD, USA). These drugs were initially dissolved in DMSO and diluted with HBSS to a concentration containing 10–25% DMSO. A volume of 100 μl of this solution containing the drug at the appropriate concentration was delivered to each mouse. Vehicle consisted of 10–25% DMSO in HBSS.

2.9. Statistics

One-way ANOVA (Randomized, block design) was used together with Scheffe or Tukey's post hoc multiple comparisons test to determine significance ($P<0.05$) between saline-and drug-treated groups. This statistical package used the GBSTAT program (Dynamic Microsystems, Silver Springs, MD, USA).

3. Results

3.1. Chronic morphine exposure suppresses CTL activity

Mice treated with morphine for 11 days exhibited significantly less SL CTL activity compared to vehicle-treated controls (Fig. 1). PL CTL activity was also significantly lower in the chronic morphine-treated mice compared to vehicle-treated controls (Fig. 2). However, both vehicle- and chronic morphine-treated mice showed similar levels of splenic NK activity (Fig. 3). SL from chronic morphine- and vehicle-treated mice were also assayed for lysis of a histoincompatible target. Neither population of SL showed any measurable cytolytic activity against ^{51}Cr-labeled P815 cells (data not shown). In addition, SL from unprimed (non-immunized) mice had no measurable CTL activity to the ^{51}Cr-labeled EL-4 targets (data not shown).

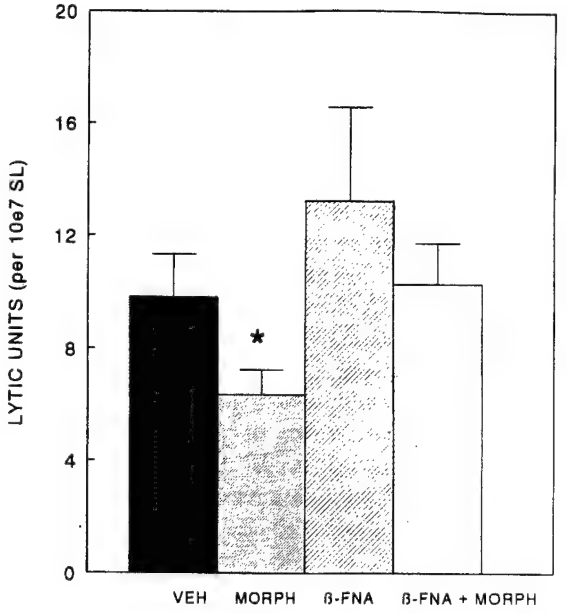

Fig. 1. β-FNA reverses morphine-induced suppression in splenic lymphocytes CTL activity. C3H/HeN mice ($n=8$/group) were administered β-FNA (40.0 mg/kg, s.c.) or vehicle 18–24 h prior to receiving morphine (50.0 mg/kg, s.c.) followed by alloimmunization (1×10^7 C57BL/6J splenocytes, i.p.) 2 h later. Mice received morphine (50.0 mg/kg, s.c.) or vehicle daily for the next 10 days. In addition, mice received β-FNA (40.0 mg/kg, s.c.) every 72 h. Mice were re-immunized (1×10^7 C57BL/6J splenocytes, i.p.) 6 days following the initial immunization. The animals were killed on day 11 and their splenic lymphocytes (SL) assayed for CTL activity using ^{51}Cr-labeled EL-4 cells. Bars represent SEM, $n=8$. *$F(3,31)=1.9646$, $P=0.05$ comparing vehicle- to chronic morphine-treated group as determined by ANOVA and Tukey's multiple comparison test.

Using cell separation techniques, the SL CTL derived from vehicle-and drug-treated mice (Fig. 4) were found to be CD8$^+$ (Table 1). CD8$^+$-enriched effector cells were also tested against the NK-sensitive target YAC-1 and found not to lyse these cells, indicating the antigen-specificity of the enriched effector cells (data not shown). Moreover, enrichment enhanced the difference in CTL activity between SL obtained from vehicle- and chronic morphine-treated mice (Table 1).

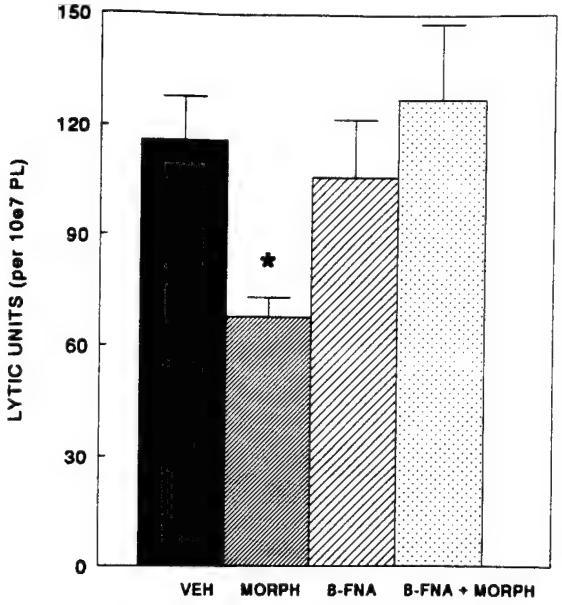

Fig. 2. Long-acting μ-antagonist β-FNA blocks morphine-induced suppression of CTL activity of peritoneal lymphocytes. Mice were treated as described in the legend to Fig. 1. Peritoneal lymphocytes (PL) were collected and assayed for CTL activity using ^{51}Cr-labeled EL-4 cells. PL CTL activity from chronical morphine-treated mice was significantly suppressed relative to other treatment groups. β-FNA co-treatment completely reversed this effect. *$F(3.18) = 3.9754$, $P < 0.05$ comparing chronic morphine-treated animals to all other groups of mice as determined by ANOVA followed by Scheffé multiple comparison test. Bars represent SEM, $n = 7$.

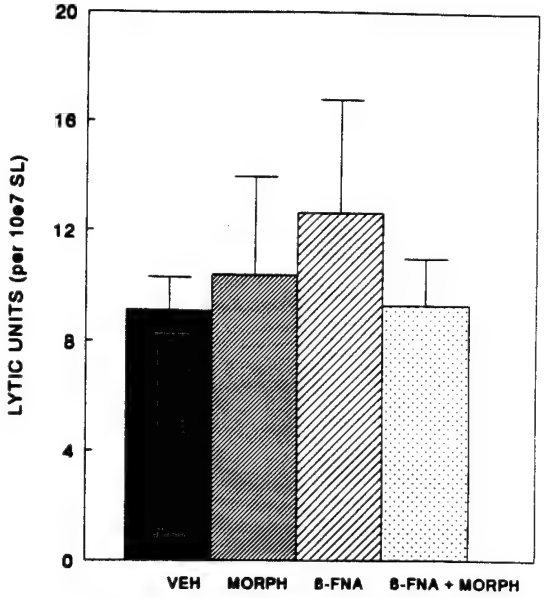

Fig. 3. β-FNA alone or in combination with morphine had no effect on splenic NK activity. Mice were treated as described in the legend to Fig. 1. Splenic lymphocytes (SL) were collected and assayed for NK activity using ^{51}Cr-labeled YAC-1 cells. Bars represent SEM, $n = 8$.

Table 1

Splenic CTL effector cells are CD8$^+$ [a]

Treatment	Unfractionated	CD4$^+$-enriched	CD8$^+$-enriched
Vehicle	17.7 ± 1.9[b]	1.8 ± 1.4	74.0 ± 6.4
Morphine	8.0 ± 1.6	1.6 ± 1.5	15.2 ± 5.3

[a] SL from vehicle- and chronic morphine-treated mice ($n = 3$/group) were enriched for either CD4$^+$ or CD8$^+$ cells (see Materials and Methods). Prior to enrichment, SL were assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. Cells from each treatment group were pooled and subsequently enriched for CD4$^+$ or CD8$^+$ cells, which subsequently were assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. Between 80–90% of SL were lost as a result of the enrichment step which is consistent with the fact that SL contain 8–12% CD8$^+$ cells (Fig. 4A). This table is a summary of two independent experiments with similar outcomes.

[b] Numbers are in LU ± SEM, $n = 3$.

3.2. β-FNA attenuates opioid-induced suppression of SL and PL CTL activity in chronic morphine-treated mice

To determine opioid receptor involvement in morphine-mediated suppression of CTL activity, studies were carried out using the μ-selective opioid antagonist β-FNA in morphine-treated animals. Pretreatment of mice with β-FNA completely blocked morphine-induced suppression of SL (Fig. 1) and PL (Fig. 2) CTL activity. β-FNA alone had no effect on SL (Fig. 1) or PL (Fig. 2) CTL activity. β-FNA alone nor in combination with morphine had any effect on splenic NK activity (Fig. 3).

3.3. β-FNA blocks morphine-induced increases in the percentage of CD4$^+$ and CD8$^+$ splenic lymphocytes

Since morphine decreased the SL and PL CTL activity and splenic effector cells mediating antigen-specific CTL activity were defined as CD4$^-$CD8$^+$, morphine exposure might modify the number of

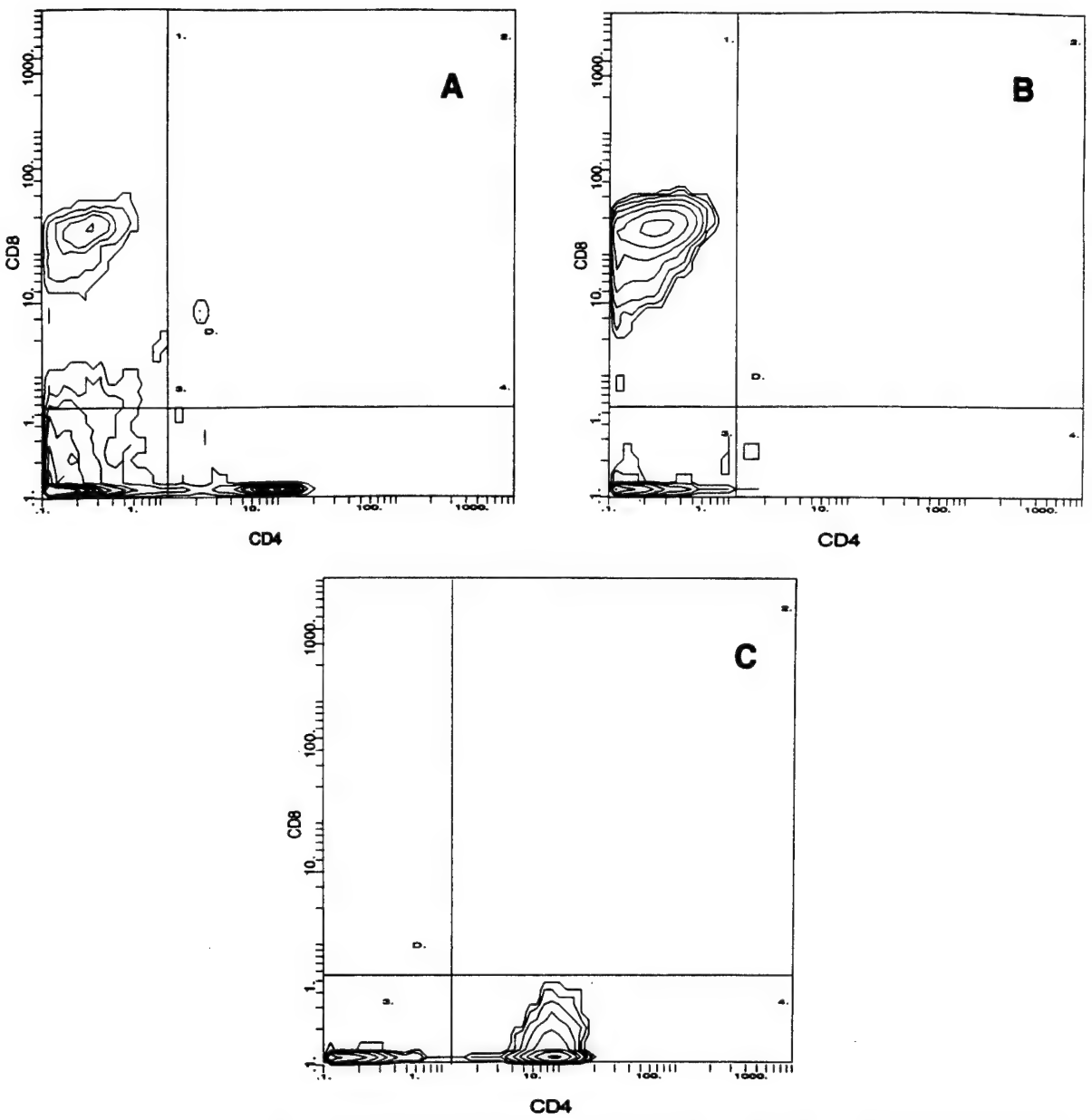

Fig. 4. Cytometric analysis of $CD4^+$- and $CD8^+$-enriched splenic lymphocytes. Splenic lymphocytes obtained from mice treated as described in the legend to Fig. 1 were labelled using anti-CD4 antibody conjugated to FITC and anti-CD8 antibody conjugated to PE. (A) Splenocytes prior to enrichment. (B) Splenocytes following $CD8^+$ enrichment. (C) Splenocytes following $CD4^+$ enrichment.

lymphocytes in the spleen. Studies were undertaken to assess T_{helper} ($CD3^+CD4^+$), $T_{cytotoxic}$ ($CD8^+$) and B (Ig^+) splenic and peritoneal lymphocyte populations from vehicle and chronic morphine-treated mice in the presence and absence of β-FNA. Phenotypic analysis of SL populations revealed an increase in the percentage of $CD3^+CD4^+$ and $CD8^+$ but not Ig^+ cells in the chronic morphine-treated mice (Fig. 5). Pretreatment of mice with β-FNA blocked the effects of morphine on the

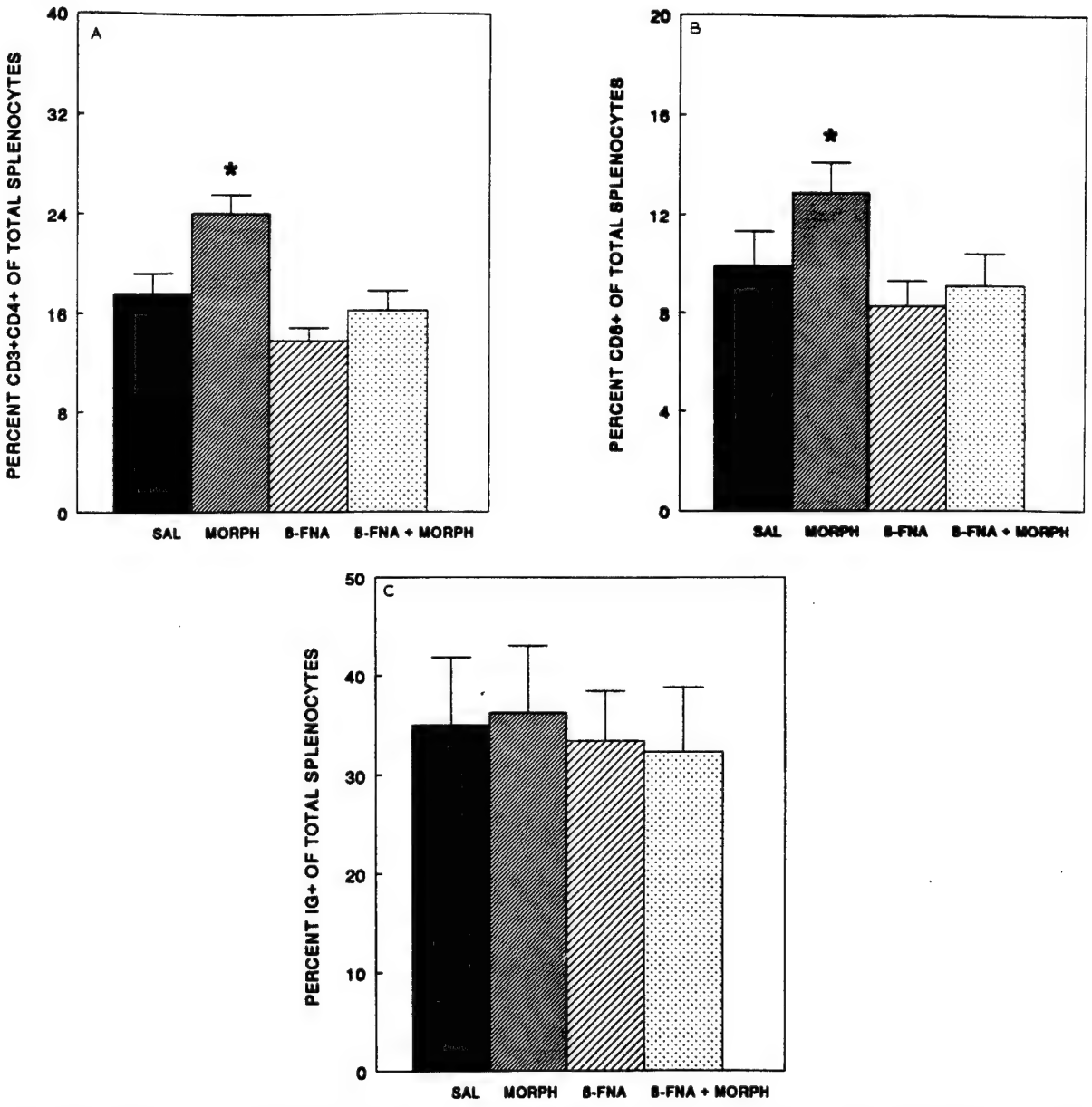

Fig. 5. β-FNA antagonizes morphine-mediated increases in splenic $CD3^+CD4^+$ and $CD8^+$ subpopulations. Mice were treated as described in the figure legend to Fig. 1. (A) β-FNA antagonized the increase in the percentage of $CD3^+CD4^+$ splenic lymphocyte following chronic morphine administration. $*F(1,6) = 4.9923$, $P < 0.05$ comparing vehicle- to chronic morphine-treated mice as determined by ANOVA and Scheffé multiple comparison test. All other groups were not significant compared to vehicle-treated animals. Bars represent SEM, $n = 7$. (B) β-FNA antagonized the increase in the percentage of $CD8^+$ splenic lymphocytes following chronic morphine administration. $*F(3,15) = 7.4202$, $P < 0.05$ comparing chronic morphine-treated mice to all other groups as determined by ANOVA and Tukey's t-test). Bars represent SEM, $n = 6$. (C) Chronic morphine exposure had no effect on the percentage of Ig^+ splenic lymphocytes. Bars represent SEM, $n = 7$.

percentage shifts in the SL population (Fig. 5) although β-FNA alone had no effect.

Changes in the percentage of lymphocyte populations in the spleen may also alter lymphocyte

Table 2

β-FNA partially antagonizes morphine-mediated augmentation of the SL proliferative response to PWM[a]

Treatment	Counts per minute ± SEM
Vehicle	14542 ± 588
Morphine	20869 ± 611**
β-FNA	13715 ± 83
Morphine + β-FNA	17622 ± 644**,*

[a] SL obtained from mice (n = 8/group) treated as described in the legend to Fig. 5 were cultured in the presence of PWM for 48 h. 200 nCi of [^{3}H]TdR was added to the wells and cells were incubated an additional 12 h. Cells were harvested and assayed for [^{3}H]TdR incorporation.

* $P<0.05$ comparing SL from mice co-administered β-FNA + morphine to chronic morphine-treated animals as determined by ANOVA and Scheffé multiple comparison test.

** $F(3,21) = 63.1663$, $P<0.01$ comparing SL from chronic morphine-treated mice to vehicle-treated controls as determined by ANOVA and Scheffé multiple comparison test.

responsivity to antigen (as shown in the CTL activity) or mitogen through the absence of appropriate cytokines necessary to drive lymphocyte growth and differentiation. Accordingly, SL from the treated groups of animals were also evaluated for proliferation to PWM. SL from chronic morphine-treated mice showed a significant increase in response to PWM compared to splenocytes from saline-treated controls (Table 2). Pretreatment of mice with β-FNA partially antagonized the effect of morphine. Pretreatment with β-FNA alone had no appreciable effect on PWM-stimulated SL proliferation (Table 2).

4. Discussion

In the present study, chronic exposure to morphine resulted in a lower response to alloimmunization as reflected by CTL activity. Since the reduced CTL activity was evident in both PL and SL populations, we speculated that a systemic pathway is involved. The results of previous studies indicate that 72-h exposure to morphine activates the HPA axis resulting in the elevation in serum corticosterone levels (Bryant et al., 1991). Endogenous corticosterone is selectively immunosuppressive (Stein and Miller, 1993) and may be partly responsible for the effects seen in the present study. However, recent data may indicate otherwise. Specifically, corticosterone levels have been measured in the chronic (11 day) morphine-treated mice following the killing of the animals and found to be reduced in comparison to the levels from vehicle-treated mice (Carpenter et al., 1994). However, this observation does not rule out the role elevated levels of corticosterone may have earlier in the immune response as indicated by others (Bryant et al. 1987; 1990; 1991; Fuchs and Pruett, 1993).

Adrenergic pathways have also previously been shown to be involved in morphine-induced immunosuppression of SL (Carr et al., 1993, Fecho et al., 1993). Interestingly, the immunosuppression following acute morphine administration is compartment specific (Bayer et al., 1990; Baddley et. al., 1993; Lysle et al., 1993). The compartmentalized nature of morphine-mediated immunomodulation may in part lie with the neuroendocrine systems innervating the immune organ (Felten et al., 1985), as well as the state of activation of the lymphocytes. Specifically, although previous work indicates lymphocytes possess μ-type opioid binding sites (Madden et al., 1987; Radulescu et al., 1991), recent studies indicate activation upregulates the expression of the morphine-sensitive binding site (Roy et al., 1992). However, morphine (10^{-5}–10^{-11} M) effects on the generation of CTLs in one-way MLCs in vitro have resulted in no discernable differences to vehicle-treated controls (unpublished observation); this observation indicates the absence of a direct effect of morphine on lymphocytes relative to CTL activity. Moreover, a previous study showed morphine-mediated immunoregulation did not correlate with circulating levels of the drug (Bryant et al., 1988). Collectively, these results suggest morphine-mediated immunomodulation following acute or chronic application of drug in vivo acts in part through neuroendocrine pathways other than the HPA axis as most recently illustrated (Hernandez et al., 1993; Carr et al., 1994a).

The pretreatment of mice with the irreversible μ-selective opioid receptor antagonist, β-FNA (Ward et al., 1982), effectively blocked the suppression of PL and SL CTL activity precipitated by chronic morphine treatment. Similar findings have also been reported for splenic NK activity following

acute morphine administration (Band et al., 1992; Carr et al., 1993). The selected dose of β-FNA was chosen based on previous data showing pretreatment of mice with β-FNA blocks [D-Ala^2, ME-Phe^4, gly(ol)^5]enkephalin- but not [D-Pen^2, D-Pen^5]enkephalin-induced analgesia in mice (Paul et al., 1989). Another recent study has shown the δ-selective opioid receptor antagonist (E)-7-benzylidine-7-dihydronaltrexone (BNTX; Porteghese et al., 1992) does not block morphine-mediated suppression of SL or PL CTL activity (Carr and Carpenter, data not shown), suggesting μ- but not δ-opioid receptor involvement.

Morphine-mediated suppression of CTL activity in alloimmunized mice may be due to immune dysfunction at the cellular level. Serine esterases, such as the BLT esterase, have been identified in CTLs (Pasternack and Eisen, 1985), are released after specific target cell binding (Pasternack et al., 1986), and have been localized to the cytotoxic granules (Young et al., 1986) implicating these enzymes as a mechanism of target cell lysis by CTLs. Other studies have indicated that serine esterases are not necessary for target cell lysis depending on the target (Trenn et al., 1987; Ostergaard et al., 1987). By inhibiting serine esterase release from CTL clones using cyclosporine A, another laboratory has hypothesized the existence of a cyclosporine-sensitive capacity to induce target cell lysis and a cyclosporine-insensitive mechanism of inducing lysis of target cells that does not require granule exocytosis (Lancki et al., 1989). Recently, a third mechanism of target cell lysis by CD4$^+$ and CD8$^+$ T cells has been identified which predominately involves direct TNF-α-dependent lysis of TNF-α-sensitive targets (Smyth and Ortaldo, 1993). A recent study showed serine esterase release and total cell serine esterase content was reduced in SL taken from chronic morphine-treated mice compared to vehicle-treated controls (Carpenter et al., 1994). In addition, no differences were found in the number of SL conjugating with targets, suggesting the conjugating process of CTLs generated over the course of 11 days was similar between vehicle- and chronic morphine-treated animals (Carpenter et al., 1994). The suppression of serine esterase release and total serine esterase content of lymphocytes from chronic morphine-treated animals is consistent with a role for this enzyme in lysing target cells. Similar results have also been obtained using IL-2- and IL-12-stimulated human CD8$^+$ T cells (Mehrotra et al., 1993). In support of this hypothesis, SL CTLs from vehicle- and chronic morphine-treated mice lyse the IL-4 targets through a Ca^{2+}-dependent process (Carr and Carpenter, data not shown) implicating granzyme A (contains serine esterases) in the 'lethal hit' (Berke, 1994). Consequently, one explanation for the reduction in cytolytic capacity of lymphocytes chronically exposed to morphine in vivo is a reduced capacity to produce granules which contain serine esterases and/or an inability to exocytosis esterase-containing granules.

In the present study, chronic morphine exposure was found to elevate the percentages of both CD4$^+$ and CD8$^+$ cells in the spleen. SL Ig$^+$ cell numbers were not affected. These results conflict with previous data showing a time-dependent increase in the CD4$^+$ cells and decrease in the CD8$^+$ cells in the spleen (Arora et al., 1990). The results of another study revealed a decrease in both CD4$^+$ and CD8$^+$ cells (Kimes et al., 1992). The discrepancies in the results may be due to the time course of morphine treatment. In the present study, morphine administration was continued for 11 days while the other studies employed morphine pellet implants and measured T-cell subsets 72–120-h post implantation (Arora et al., 1990; Kimes et al., 1992). Another difference between these investigations involves the different strains of mice employed. It has previously been shown that there are strain differences in susceptibility to morphine effects on immune responses (Bussiere et al., 1992). Our results indicate that chronic morphine treatment increases the numbers of both CD4$^+$ and CD8$^+$ cells in the spleen. Furthermore, there is an increase in proliferation in response to PWM by SL from chronic morphine-treated mice compared to SL from vehicle-treated controls which is opposite to reports assessing mitogen-induced lymphocyte proliferation following acute morphine administration (Hernandez et al., 1994). The response to mitogen by SL from chronic morphine-treated animals may reflect changes in the population of T_H subpopulations. Additional studies using CD markers expressed on these cells are in progress. Other studies using chronic morphine-treated rhesus monkeys has shown an increase in the percentage of CD4$^+$CD29$^+$ PBMCs and a decrease

the percentage of $CD4^+CD45RA^+$ PBMCs (Carr and France, 1993). Such a shift in the memory/helper $CD4^+$ population may alter cytokine production affecting the generation of CTLs.

The data indicating that chronic morphine exposure acts selectively on antigen-driven cytolytic function and not NK cytolytic activity may implicate morphine in the regulation of cytokine production. Since CTL maturation and maintenance of function require IL-2 and in certain instances IL-6 (Bass et al., 1993), morphine regulation of the production of these cytokines could affect CTL generation and activity. In support of this hypothesis, it is known that morphine-mediated suppression of the primary antibody response is the result of a reduction in IL-6 synthesis; exogenous IL-6 attenuated the suppression in antibody production (Bussiere et al., 1993). Another study revealed that morphine pellet implantation has no effect on PMA-induced IL-2 production (Saini and Sei, 1993); it may be that morphine selectively inhibits specific cytokines. Whereas morphine may reduce the production of some cytokines, recent data indicate that it augments TGF-β production in vitro (Chao et al., 1992); this cytokine is a negative regulator of many T-cell responses (Ishizaka et al., 1992).

In summary, the data indicate that prolonged exposure to morphine diminishes the capacity to sustain CTL activity to alloantigenic cells. Since morphine (and heroin) has previously been shown to promote the growth of HIV-1 in mitogen-stimulated PBMCs in vitro (Peterson et al., 1990; Adler et al., 1993) and both reduce immune responses, the molecular mechanisms of action of opioids in affecting immune homeostasis will be a primary focus of future research.

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Morphine-Induced Suppression of Cytotoxic T Lymphocyte Activity in Alloimmunized Mice Is Not Mediated through a Naltrindole-Sensitive Delta Opioid Receptor

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Key Words
Morphine
Cytotoxic T lymphocyte
BNTX
Naltrindole
Opioid receptor
Natural killer activity

Abstract
The effect of chronic morphine exposure on natural killer (NK) activity in vivo and the generation of cytotoxic T lymphocytes (CTLs) in vitro and in vivo was investigated. Chronic exposure to morphine (10^{-5}–10^{-11} *M*) in vitro had no effect on the generation of antigen-driven effector cells. However, the daily administration of morphine (50.0 mg/kg, s.c.) into alloimmunized mice (C57BL/6 into C3H/HeN) for 11 days resulted in a decrease in peritoneal and splenic CTL activity but not splenic NK activity. In addition, there was a 60% decrease in the number of thymocytes recovered from chronic morphine-treated mice compared to vehicle-treated controls. However, the overall percentage of CD4+CD8–, CD4–CD8+ and CD4+CD8+ thymocytes did not change between the two groups of treated animals. Pretreatment of the mice with the δ_1-selective antagonist, (E)-7-benzylidine-7-dihydronaltrexone (BNTX, 0.6 mg/kg, s.c.) did not block morphine-mediated suppression of splenic CTL activity but did block morphine-induced suppression of peritoneal lymphocyte CTL activity. In addition, BNTX pretreatment alone augmented splenic NK activity and such augmentation was blocked following chronic morphine exposure. In contrast, the δ-selective antagonist, naltrindole (20.0 mg/kg, s.c.), had no effect alone nor antagonized the action of morphine on CTL activity. Splenic CTL effector cells from either treated group of animals lysed their target (EL-4 lymphoma) through a Ca^{2+}-dependent mechanism. Collectively, the results indicate morphine suppresses CTL activity through an indirect pathway, insensitive to naltrindole rather than through direct lymphocyte opioid receptors.

Introduction

The abuse of opioids (e.g. heroin and fentanyl) resulting in a compromised immune system [1–3] and a greater susceptibility to infectious agents [4, 5] has made this class of compounds a liability and predicted cofactor in the acquisition and spread of HIV-1 [6]. The implicated cofactor relationship between opioids and HIV-1 is supported by data showing morphine amplification of HIV-1 expression in phytohemagglutinin-activated peripheral

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blood mononuclear cells [7]. In addition, morphine increases lipopolysaccharide-primed microglial production of tumor necrosis factor-α which in turn promotes HIV-1 expression in latently infected promonocytes [8] and promonocyte-fetal brain cell cocultures [9]. Within the immune system, chronic morphine treatment has been shown to increase the percentage of CD4+CD29+ peripheral blood T lymphocytes in rhesus monkeys [10] which are the reported reservoirs for simian immunodeficiency virus [11]. At the molecular level, pokeweed-mitogen-stimulated peripheral blood mononuclear cells from rhesus monkeys treated chronically with morphine possess elevated levels of NFκB which is a regulatory element for key cytokines including interleukin-2 and tumor necrosis factor-α and binds to sites within the HIV-1 promoter [12]. Taken together, the data would suggest opioids increase transcriptional regulators which promote HIV-1 replication, augment the reservoir cell population for HIV-1 replication in the peripheral blood and increase cytokine production by microglial cells in the brain increasing the likelihood of replication of this virus in the central nervous system. However, the effector cell population (cytotoxic T lymphocytes, CTL) which is typically responsible for monitoring virus infection has only recently been evaluated in the presence of opioids.

Previous studies have shown the endogenous opioid peptides β-endorphin and [met]-enkephalin augment the generation of CTLs in one-way mixed lymphocyte cultures (MLCs) [13]. More recently, alloimmunized mice chronically treated with morphine (50.0 mg/kg, s.c., daily for 11 days) presented with significantly suppressed CTL activity in splenic and peritoneal lymphocyte populations [14]. Moreover, the μ-selective opioid receptor antagonist, β-funaltrexamine, blocked the suppression of CTL activity in the chronic morphine-treated mice, suggesting the effect was mediated in part by μ opioid receptors [15]. The mechanism of this suppression was identified to include a decrease in the production and release of serine esterases which are typically associated with the 'lethal hit' by a proportion but not all CTLs [14]. The release of granules (granzyme A) containing the serine esterases is a Ca^{2+}-dependent phenomenon while the other mode of CTL-directed lysis of target cells involves receptor-mediated apoptosis which does not require extracellular Ca^{2+} [16].

Therefore, we investigated the initial observations of morphine-mediated suppression of CTL activity: (i) the potential involvement of δ opioid receptors; (ii) the calcium-dependent nature of CTL-directed cytolysis, and (iii) T cell precursor development through the assessment of thymic subpopulations in chronic morphine-treated mice.

Materials and Methods

Mice and Tumor Lines

Female C57BL/6 and C3H/HeN (Harlan Sprague Dawley, Indianapolis, Ind., USA) mice (6–7 weeks of age) were housed in groups of 5 per cage and maintained on a 12-hour light/dark cycle. Access to water and food (Purina Mouse Chow) was available ad libitum. The YAC-1 and EL-4 mouse lymphoma cell lines and P815 mastocytoma cell line were originally obtained from the American Type Culture Collection (Rockville, Md., USA); the cells have been maintained in culture by biweekly passage over a 6-month period. All animals used in these studies were maintained in accordance with the Committee on the Use and Care of Animals, Louisiana State University Medical Center, and the guidelines of the Committee on Care and Use of Laboratory Animals Resources, National Research Council, Department of Health, Education, and Welfare Publications Number (National Institutes of Health) 85-23 revised 1985.

Morphine Treatment Regimen

A dose-response study has established that 50.0 mg/kg of morphine s.c. result in maximal suppression of cytolytic activity [17; unpubl. observation]. Consequently, this dose was used in the in vivo experiments.

C3H/HeN mice (n = 10/group) were administered vehicle or the δ opioid receptor antagonists BNTX (0.6 mg/kg, s.c.) or naltrindole (20.0 mg/kg, s.c.) 30 min prior to the initiation of the chronic morphine treatment. Morphine was administered 2 h prior to receiving 1×10^7 C57BL/6 spleen cells, i.p. Following the immunization, mice received vehicle or morphine daily for an additional 6 days. On day 7, mice were reimmunized with 1×10^7 C57BL/6 spleen cells, i.p., 2 h after the administration of vehicle or morphine. Following the second immunization, mice received morphine or vehicle daily for an additional 3 days. In addition to the daily administration of morphine or vehicle, mice received either vehicle, naltrindole (20.0 mg/kg, s.c.) or BNTX (0.6 mg/kg, s.c.) daily 30 min prior to morphine administration. On day 11 after the initial immunization, the mice were sacrificed and peritoneal (PL) and splenic lymphocytes (SL) were assayed for CTL and NK activity. The concentration of BNTX used in this study was found to be fully antagonistic to [(D-Pen^2, D-Pen^5)enkephalin] in mice [18]. Likewise, the concentration of naltrindole used in this study has previously been shown to be selective for antagonizing δ but not μ or κ opioid receptors [19]. In addition, splenic lymphocytes generated in this manner have previously been shown to be antigen-specific, CD8+CD4– effector cells [14, 15].

Lymphocyte and Thymocyte Preparation

All mice were sacrificed by CO_2 asphyxiation and peritoneal lavage was performed using 10 ml of sterile Hanks' balanced salt solution (HBSS). Cells were collected by recovering 10 ml of peritoneal fluid through a 20-gauge needle and 10-ml syringe. Thymus and spleens were removed and cell suspensions were prepared by mechanical dispersion. SL, PL and thymocytes were washed with HBSS (250 *g*, 5 min). Red blood cells were osmotically lysed using 0.84% NH_4Cl; the cells were subsequently washed with HBSS (250 *g*, 5 min)

and resuspended in RPMI-1640 containing 10% fetal calf serum and 2.5% Hybri-max (Sigma, St. Louis, Mo., USA) antibiotic/antimycotic solution (complete media). Cells were counted and examined for viability via trypan blue exclusion dye.

In vitro Generation of Cytotoxic Effector Cells

Sterile suspensions of SL were prepared, and red blood cells were osmotically lysed as described above. In vitro cultures were established in sterile flat-bottomed 24-well microtiter plates (Costar, Cambridge, Mass., USA) with complete media. C3H/HeN SL (6×10^6 cells) were cocultured for 72–120 h with irradiated (900 rad) stimulator (C57BL/6) cells (4×10^6 cells) in the presence or absence of morphine (10^{-5}–10^{-11} *M*). The indicated amount of morphine was added daily to the cultures. These cultures were incubated in 5% CO_2 atmosphere at 37° C. At the end of the culture period, cells were pelleted (200 *g*, 5 min), washed with HBSS and resuspended in a volume of complete media to give the effector-to-target cell ratios of 40:1, 20:1, 10:1 and 5:1. Each effector-to-target cell ratio was determined in triplicate. The percent killing at each effector-to-target ratio was converted to lytic units (LU). One LU was defined as the number of effector cells able to lyse 30% of the targets (EL-4 lymphomas) and this unit was expressed per 10^7 cells.

^{51}Cr-Release Cytolytic Assay

SL and PL CTL activity was assayed using a 4-hour microcytotoxicity assay with ^{51}Cr-labeled EL-4 cells (H-2^b) or P815 cells (H-2^d) as targets. ^{51}Cr-labeled YAC-1 lymphoma cells were used as targets to measure SL NK activity. Between 2×10^4 and 100×10^4 effector cells were mixed with 1×10^4 target cells in conical 96-well microtiter plates (Costar) in a reaction volume of 0.2 ml of complete media. The cultures were incubated 4 h at 37° C in a 5% CO_2 atmosphere. A 100-µl aliquot of cell-free supernate was taken from each well and its ^{51}Cr content was determined using a Beckman γ-counter. The cytolytic activity was determined as follows: percent cytolytic activity = [(experimental ^{51}Cr release – spontaneous ^{51}Cr release)/(total cell-associated ^{51}Cr release – spontaneous ^{51}Cr release)] × 100, where 'spontaneous' refers to ^{51}Cr release by target cells in the absence of effector cells. Total cell-associated ^{51}Cr was determined by measuring the ^{51}Cr in the supernate of target cells lysed with 0.1% Triton X-100 in complete medium. Each effector-to-target cell ratio was measured in triplicate/animal. The percent lysis was then converted to LU. One LU was defined as the number of SL or PL able to lyse 20% of the target cells, and this unit was expressed per 10^7 cells. To determine antigen specificity for the in vitro generated CTL assay, P815 mastocytoma cells were ^{51}Cr-labeled and used as targets in the 4-hour microcytotoxicity assay. To determine the Ca^{2+} requirement in the CTL-directed cytolysis of the target cell, SL CTLs were incubated in the presence or absence of 3.6 m*M* EGTA during the 4-hour microcytotoxicity assay.

FACS Analysis of Thymocyte Populations

Thymocytes from the vehicle- or drug-treated groups of mice were collected and washed in RDF buffer (R & D Systems, Minneapolis, Minn., USA) and resuspended in 20 µl of RDF buffer containing fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 (IgG2b isotype, Gibco BRL, Gaithersburg, Md., USA) and phycoerythrin (PE)-conjugated rat anti-mouse CD8 (IgG2b isotype, Gibco BRL). FITC- and PE-conjugated rat IgG2b were used as isotypic controls. The cells were allowed to incubate for 20–25 min on ice in the dark. 900 µl RDF buffer were added to the cells which were then centrifuged (250 *g*, 5 min). The supernatant fluid was discarded, and the cell pellet was resuspended in 250 µl of RDF buffer and 250 µl of 2% paraformaldehyde (Sigma) and analyzed by FACS for the percentage of stained cells in the cell population. Light scatter was collected at 488 nm, and the emitted light which passed through a long pass filter was analyzed at 525 nm (FITC) or 575 nm (PE) on a Coulter Elite FACS (Coulter, Hialeah, Fla., USA). 5,000 gated events were collected and analyzed per sample. Compensation between FITC and PE amounted to 20–25%.

Reagents

Morphine sulfate was generously provided by the Research Technology Branch of the National Institute on Drug Abuse (Rockville, Md., USA). The δ_1-selective opioid receptor antagonist BNTX and naltrindole were purchased from Research Biochemicals (RBI, Natick, Mass., USA). The drugs were dissolved in 10% dimethyl sulfoxide in HBSS. Vehicle consisted of 10% dimethyl sulfoxide in HBSS.

Statistics

One-way ANOVA (randomized, block design) was used together with Tukey's protected t test or Scheffé's multiple comparison tests in comparing individual means between treated groups of animals in order to determine significance ($p < 0.05$). This statistical package used the GBSTAT program (Dynamic Microsystems, Silver Springs, Md., USA).

Results

Morphine Exposure in vitro Has No Effect on the Generation of Antigen-Driven Effector Cells

One-way MLCs were set up to determine the direct effects of morphine on the generation of cytolytic effector cells. The results show morphine (10^{-5}–10^{-11} *M*) added to cultures daily had no effect on the production of cytolytic effector cells compared to vehicle-treated controls determined 72, 96 and 120 h following initiation of culture (fig. 1). Cytolytic effector cells collected on day 5 following culture were also tested for target specificity using ^{51}Cr-labeled P815 mastocytoma cells (H-2^d haplotype). No measurable cytolysis of this cell line was detected (data not shown).

Chronic Morphine Treatment Antagonizes the BNTX-Mediated Augmentation of Splenic NK Activity

C3H/HeN mice chronically administered morphine exhibited lower SL (fig. 2) and PL CTL (fig. 3) activity compared to vehicle-treated controls. Consistent with previous results, chronic morphine exposure had no effect on splenic NK activity (fig. 4). Pretreatment with BNTX antagonized morphine-induced suppression of PL CTL activity (fig. 3) but not SL CTL activity (fig. 2). Pretreatment of mice with BNTX alone had no effect on SL

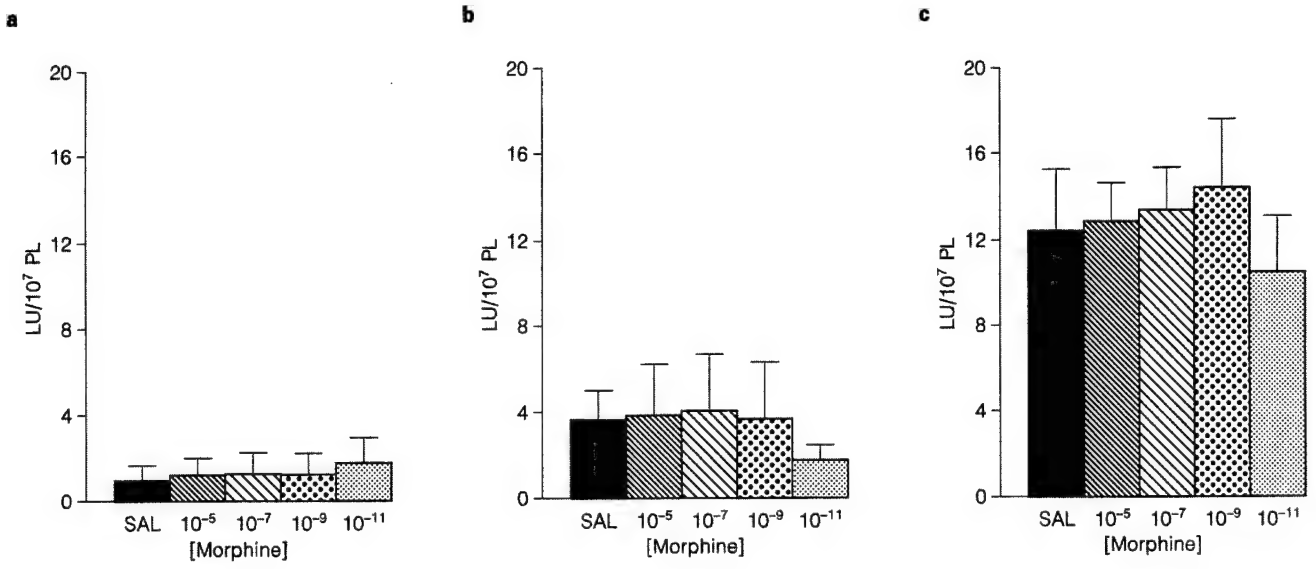


Fig. 1. Chronic morphine exposure has no direct effect on the generation of antigen-driven effector cells in one-way MLC. C3H/HeN SL (6×10^6 cells) were cocultured with irradiated (900 rad) C57BL/6 SL (4×10^6 cells) in the presence or absence of the indicated concentration of morphine added daily to each culture. Vehicle was added to control cultures. Following 72- (**a**), 96- (**b**) or 120-hour (**c**) culture incubation periods, the cells were harvested, enumerated and assayed for cytolytic activity using ^{51}Cr-labeled EL-4 cells as targets. Bars represent SEM, n = 5. SAL = Saline.

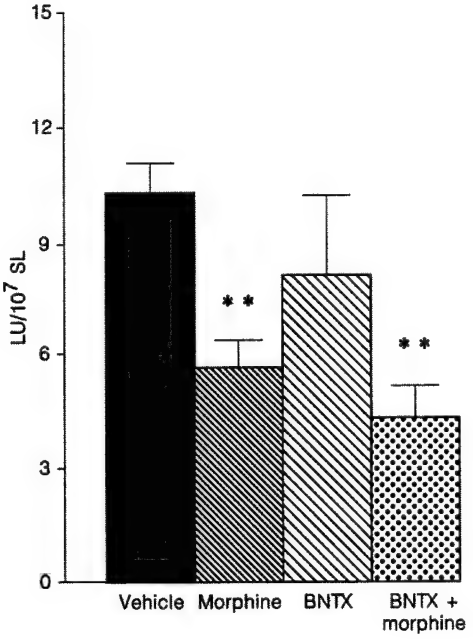
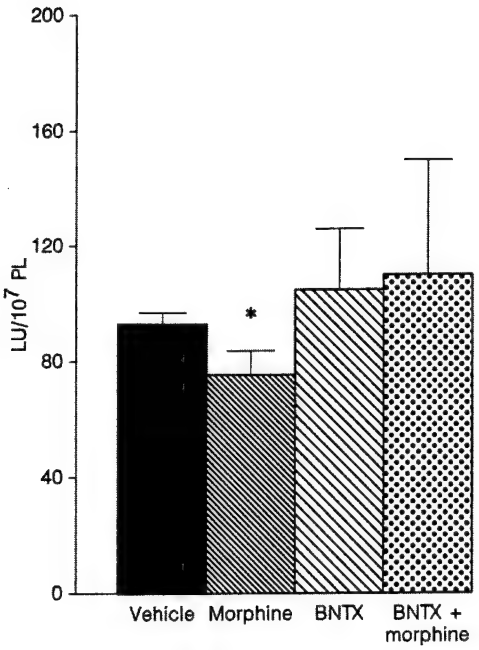


Fig. 2. BNTX does not antagonize morphine-mediated suppression of splenic CTL activity. C3H/HeN mice (n = 9/group) were administered BNTX (0.6 mg/kg, s.c.) or vehicle 30 min prior to receiving morphine (50.0 mg/kg, s.c.) or vehicle. Two hours after morphine administration, mice were alloimmunized (1×10^7 C57BL/6 splenocytes, i.p.). Groups of mice received morphine (50.0 mg/kg, s.c.), BNTX (0.6 mg/kg, s.c.), both morphine and BNTX, or vehicle daily for 10 additional days. All mice were reimmunized (1×10^7 C57BL/6 splenocytes, i.p.) 6 days after the initial immunization. Animals were sacrificed on day 11 and SL were collected and assayed for CTL activity using ^{51}Cr-labeled EL-4 cells. Chronic morphine or BNTX + morphine exposure significantly suppressed splenic CTL activity, ** $p < 0.01$ comparing drug-treated to vehicle controls as determined by ANOVA and Tukey's post t test. Bars represent SEM.



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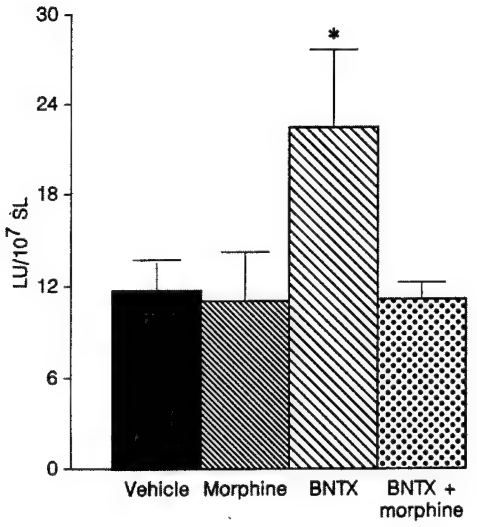

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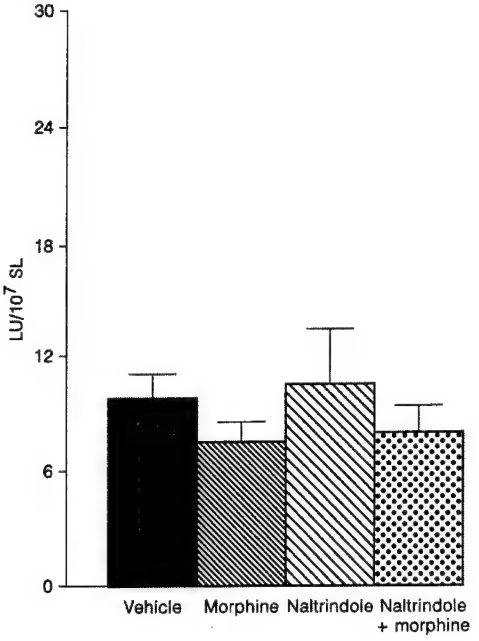

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Fig. 3. BNTX antagonizes morphine-mediated suppression of peritoneal CTL activity. Mice (n = 9/group) were treated as described in the legend to figure 2. PL were assayed for CTL activity using ^{51}Cr-labeled EL-4 cells. * p = 0.06 comparing morphine-treated to vehicle-treated controls. Bars represent SEM.

Fig. 4. Morphine antagonizes BNTX-mediated augmentation of splenic NK activity. Mice (n = 10/group) were treated as described in the legend to figure 2. SL were assayed for NK activity using ^{51}Cr-labeled YAC-1 cells. Chronic BNTX exposure to mice resulted in potentiation of splenic NK activity. When mice were coadministered morphine and BNTX, splenic NK activity maintained levels similar to vehicle- or chronic morphine-treated animals. * $p < 0.05$ comparing morphine-treated to vehicle-treated group as determined by ANOVA and Tukey's post t test. Bars represent SEM.

Fig. 5. Naltrindole has no effect alone or in combination with morphine on splenic NK activity. Mice (n = 6/group) were treated as described. SL were assayed for NK activity using ^{51}Cr-labeled YAC-1 cells. Bars represent SEM.

(fig. 2) or PL (fig. 3) CTL activity. However, BNTX treatment alone resulted in a significant increase in splenic NK activity (fig. 4). In animals chronically treated with both morphine and BNTX, no measurable increase in splenic NK activity was observed (fig. 4).

Naltrindole Does Not Antagonize Morphine-Mediated Suppression of CTL Activity

In order to determine the generality of the effects of BNTX, another δ-selective antagonist, naltrindole was investigated under the same conditions used for BNTX.

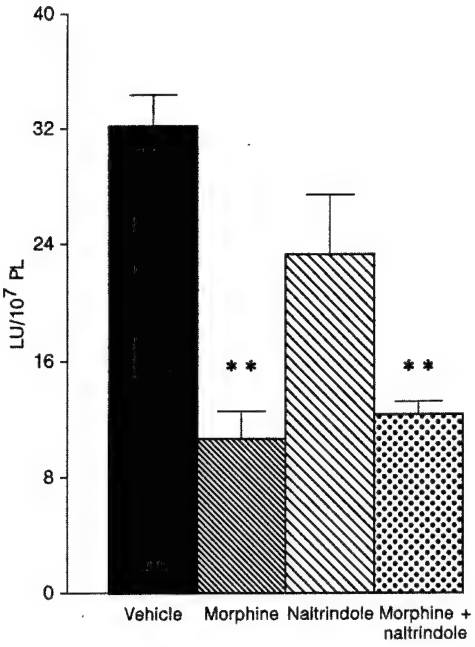

Fig. 6. Naltrindole does not antagonize morphine-mediated suppression of PL CTL activity. Mice (n = 6/group) were treated as described. PL were recovered following the sacrifice of the animals and assayed for CTL activity using ^{51}Cr-labeled EL-4 cells as targets. ** $F(3,23) = 15.4755$, $p < 0.01$ comparing morphine and morphine + naltrindole to vehicle groups as determined by ANOVA and Scheffé's post hoc multiple comparison test. Bars represent SEM.

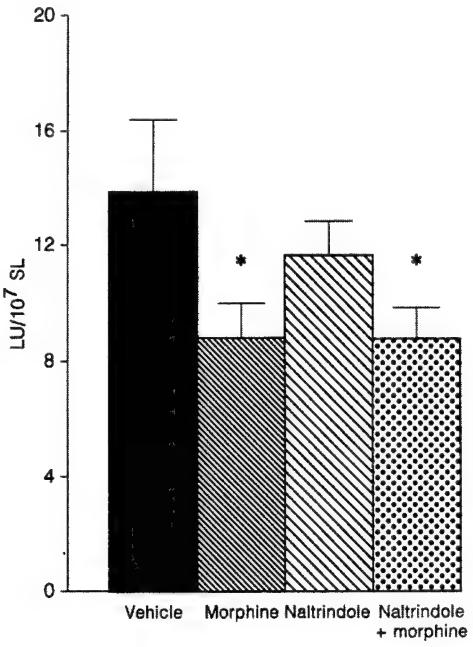

Fig. 7. Naltrindole does not antagonize morphine-mediated suppression of SL CTL activity. Mice (n = 6/group) were treated as described. SL were collected and assayed for CTL activity using ^{51}Cr-labeled EL-4 cells as targets. *$F(3,23) = 2.4238$, $p < 0.05$ comparing morphine and morphine + naltrindole to vehicle groups as determined by ANOVA and Tukey's post hoc t test. Bars represent SEM.

Unlike BNTX, naltrindole had no effect alone on SL NK activity (fig. 5) nor did it antagonize the suppressive effect mediated by morphine on PL (fig. 6) or SL (fig. 7) CTL activity. Consistent with previous observations, chronic morphine exposure was found to significantly reduce the cellularity of the spleen (table 1). Co-administration of naltrindole (20.0 mg/kg, s.c.) to mice chronically exposed to morphine (50.0 mg/kg) partially antagonized this effect (table 1).

Chronic Morphine Treatment Results in Thymic Atrophy but Not a Disproportionate Increase or Decrease in the Percentage of Thymocyte Subpopulations

Since our results indicated morphine exposure suppresses a central T cell function, we investigated the progenitor T cells found in the thymus. Chronic morphine treatment significantly reduced the absolute number of cells recovered from the thymus. Specifically, there was a

Table 1. Naltrindole partially antagonizes morphine-mediated suppression in absolute cell numbers in the spleen[a]

Treatment	Cell number (mean ± SEM)
Vehicle	$545 \pm 0.40 \times 10^7$
Morphine	$2.87 \pm 0.33 \times 10^{7*}$
Naltrindole	$6.01 \pm 0.89 \times 10^7$
Morphine + naltrindole	$4.15 \pm 0.54 \times 10^7$

[a] C3H/HeN mice (n = 6/group) were treated as described. Following sacrifice of the animals, SL were recovered and counted using trypan blue exclusion dye. Less than 2% of the cells were stained with trypan blue.

* $F(3,23) = 8917$, $p < 0.05$ comparing vehicle to morphine group as determined by ANOVA and Scheffé's post hoc multiple comparison test. All other groups were insignificant ($p > 0.05$) relative to vehicle counts.

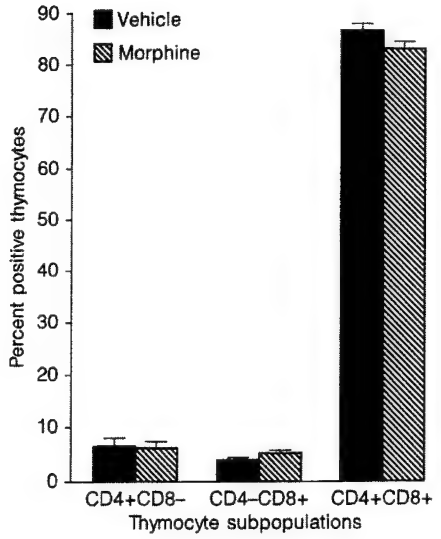

Fig. 8. Chronic morphine exposure does not modify the percentage of CD4+CD8–, CD4–CD8+ or CD4+CD8+ thymic subpopulations. Mice (n = 6/group) were treated as described in the legend to figure 2. Upon sacrifice of the animals, thymocytes were collected, labeled as described and analyzed by FACS. Bars represent SEM.

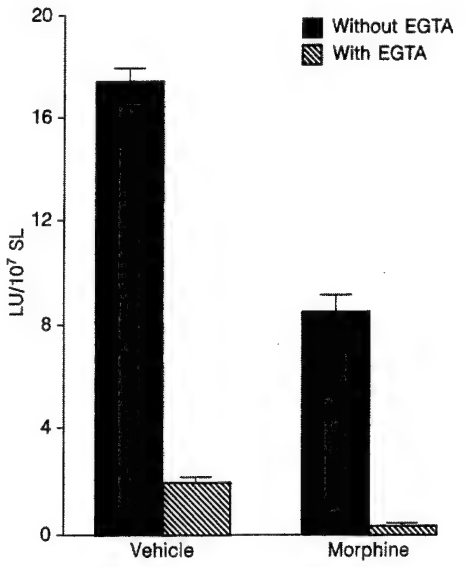

Fig. 9. SL CTL activity is Ca^{2+} dependent. SL obtained from either vehicle- or chronic morphine-treated mice (n = 3/group) were assessed for a Ca^{2+} requirement during the 'lethal hit' by adding 3.6 m*M* EGTA to the media during the 4-hour microcytotoxicity assay. Bars represent SEM.

60% decrease in recovered cells from chronic morphine-treated mice ($5.5 \pm 0.6 \times 10^7$ thymocytes) compared to vehicle-treated controls ($13.38 \pm 0.98 \times 10^7$ cells) ($p < 0.01$, ANOVA and Scheffé). However, the percentage of CD4+CD8–, CD4–CD8+ and CD4+CD8+ cells within the thymus did not change (fig. 8).

CTLs Generated in the Alloimmunized C3H/HeN Mice Lyse Their Targets through a Ca^{2+}-Dependent Mechanism

There are currently two proposed mechanisms of lymphocyte-driven cytotoxicity: (i) nonsecretory and (ii) secretory [16]. CTLs operate through either the Ca^{2+}-dependent membranolytic pathway [20] or a receptor-driven apoptotic-inducing mechanism which does not involve extracellular Ca^{2+} [16]. To identify which mechanism of CTL-directed cytolysis of target cells was utilized by the effector cells, SL were assayed for cytolysis of EL-4 target cells in the presence or absence of EGTA. The results show SL from morphine- and vehicle-treated animals lyse their targets through a Ca^{2+}-dependent process (fig. 9).

Discussion

Consistent with previous findings, the present study shows chronic treatment of mice with morphine resulted in a lower immune response to alloimmunization as reflected by CTL activity in both PL and SL populations. Pretreatment of mice with the δ_1-selective opioid receptor antagonist BNTX [21] did not block morphine-induced suppression of SL CTL activity but did antagonize morphine-mediated suppression of PL CTL activity. However, naltrindole did not antagonize morphine-mediated suppression of SL or PL CTL activity. The data suggest morphine-mediated suppression of SL CTL activity is not mediated through δ opioid receptors. However, the results showing BNTX antagonizes morphine-mediated suppression of PL CTL activity similar to β-funaltrexamine seems to suggest either both δ_1 and μ opioid receptors are involved either centrally (brain) or peripherally (spinal) or BNTX exhibits a peculiar activity not normally associated with δ opioid receptor antagonists. In support of the former notion, naltrindole has recently been shown to antagonize the δ_2-selective opioid agonist [D-Ser^2, Leu^5,

Thr[6]] enkephalin- but not the δ_1-selective opioid agonist [D-Pen[2], D-Pen[5]]enkephalin-mediated analgesia, even though naltrindole blocks both agonist binding to brain δ opioid receptors suggesting the naltrindole functionally antagonizes δ_2 opioid receptor events [22].

The data indicating that morphine blocks the augmentation in splenic NK acitivity following chronic BNTX administration seems to suggest μ and δ opioid receptors may share a common pathway distal to the opioid binding site. The level of this interaction may be related to the level of action of these compounds. BNTX has been shown to act spinally through δ_1-type opioid receptors [18] which may also include μ_1 opioid receptors, while morphine binds preferentially to μ_2 opioid receptors [23]. Although μ and δ opioid ligands have different sites of action within the central nervous system relative to inducing or antagonizing analgesia, the relationship of the opioid receptor types on immunocompetence in vivo has not been determined. Recently, two studies suggest opioid-mediated analgesia and immunomodulation are functionally independent. Specifically, the administration of morphine into the anterior hypothalamus has been shown to inhibit blood lymphocyte proliferation but has no measurable analgesic action [24]. In a second study, mice pretreated with naltrexone (10.0 mg/kg, s.c.) and subsequently administered increasing increments of morphine up to 100.0 mg/kg showed appreciable analgesia (50–60% of maximal effect) but no suppression of splenic NK activity [17]. Taken together, it is tempting to speculate the existence of opioid receptors which can be distinguished by analgesic versus immunomodulatory activities. This being the case, it may be possible to identify an opioid compound which induces analgesia without the immunosuppressive side effects. Recently, one such compound, OHM3295, a fentanyl derivative, has been found to induce analgesia without suppressing splenic NK activity through a naltrexone-sensitive pathway [25]. In fact. OHM3295 augmented splenic NK activity in a dose-dependent fashion. Consequently, future work is necessary to determine the action of central (supraspinal) and peripheral (spinal) opioid receptors relative to analgesia and immunocompetence.

The present investigation shows that mice administered BNTX daily over 11 days had elevated levels of splenic NK activity. This increase could be due to a redistribution of NK cells from the circulation into the spleen or the activation of pre-NK cells to fully competent cytolytic cells. The observation that mice chronically treated with an opioid antagonist have elevated levels of splenic NK activity is not a novel finding. A previous study has shown mice chronically treated (168 h) with naloxone (0.1–1.0 mg/kg, s.c.) displayed increased levels of splenic NK activity compared to vehicle- or (+)-naloxone-treated control mice [26]. Taken together, the results imply endogenous opioid pathways are important in the regulation of immune homeostasis.

In the present study, SL CTL effector cells incubated with EGTA during the ^{51}Cr-release microcytotoxicity assay did not exhibit cytolytic activity suggesting a requirement for Ca^{2+}. These results complement previous work showing SL from morphine-treated mice possess significantly lower levels of serine esterases [14] which are utilized in the Ca^{2+}-dependent, secretory CTL-directed lysis of target cells [16]. Therefore, the membranolytic pathway used by effector cells generated in the alloimmunized C3H/HeN mice is altered following chronic morphine treatment. Currently, it is not known at what level the modification in the membranolytic pathway is affected by morphine. However, an aberrant response in the generation of cAMP following exposure to alloantigen by CD8+-enriched effector cells taken from morphine-treated animals has been reported [14].

Morphine-mediated suppression of CTL activity is not simply due to a direct interaction of drug with lymphocytes. Although lymphocytes have been shown to possess opioid receptors [27] and recently, an orphan opioid receptor has been cloned and sequences from murine splenic lymphocytes [37], morphine was found to have no effect on the generation of CTLs in one-way MLCs. Therefore, similar to morphine-mediated suppression of splenic NK activity, morphine-induced suppression of CTL activity is indirect, potentially involving the hypothalamic-pituitary adrenal axis [28] and/or the sympathetic nervous system [29, 30].

The occurrence of thymic atrophy following morphine exposure has previously been described by numerous laboratories [28, 31, 32]. In one investigation, the administration of morphine resulted in a time-dependent decrease in the CD4+CD8+ thymocyte population which recovered to normal levels by day 10 [32]. Consistent with these findings, our results show no change in the percentage of total double-positive thymocytes following the sacrifice of mice on day 11. The relationship between the initial decrease in thymocyte population following morphine exposure and the generation of CTLs in the spleen and peritoneum is currently unknown. However, observations showing elevated levels of CD4+ and CD8+ SL in the chronic morphine-treated mice [14] suggest an overcompensation in the peripheral T cell population. This overcompensation might, in part, be due to the inability of the

immune system from the morphine-treated mice to clear the antigen. The clearance defect could be the result of dysfunctional cytolytic activity mediated by the CTL effector cells as reported in the present study, inappropriate processing and presentation of antigen to pre-CTLs by macrophages, or a combination of both processes. Previous studies indicate a decrease in the index of phagocytosis by peritoneal and splenic macrophages following morphine exposure substantiating the notion of a clearance defect [33–35].

To determine the effect of the frequency of exposure to morphine in alloimmunized mice on CTL and NK activity, a recent study has shown a single exposure to morphine (50.0 mg/kg, s.c.) 2 h prior to alloantigen immunization can significantly reduce (40–50%) PL but not SL CTL activity through a naltrexone-sensitive pathway [Carr et al., submitted]. However, a subchronic exposure (daily for 5 days) to morphine (50.0 mg/kg, s.c.) was not found to modify CTL activity in alloimmunized mice suggesting morphine effects on CTL activity are elicited through a complex cascade of events which have not been elucidated.

In summary, the present study indicates the repeated exposure to morphine over an extended period of time (daily for 11 days) has detrimental consequences on CTL activity. It is tempting to speculate that this diminution in effector cell function may in part contribute to the elevated risk among opioid abusers for the acquisition of viral infections. Controlled, experimental studies have shown mice exposed to morphine succumb to viral infections earlier and in greater numbers than vehicle-treated controls [14, 36]. These findings are consistent with published results in the human population [4, 5] supporting the supposition of opioids as cofactors for viral infections including AIDS [6]. Future work is required to identify the intracellular signalling pathways of effector cells modified by opioids following in vivo exposure as well as the neuroendocrine signals elicited by morphine either centrally or peripherally which ultimately affect the immune system. Pending the outcome of these findings, pharmacological intervention seems possible either through the development of novel analgesics which do not activate those neuroendocrine pathways involved in immunomodulation or chemical antagonists which block the opioid-induced immunosuppression but not the analgesia.

Acknowledgments

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COOPERATIVE AGREEMENT NO.: DAMD17-93-V-3013

TITLE: NEURAL RESPONSE TO INJURY, PREVENTION, PROTECTION AND REPAIR

CHAPTER: 6B; NEUROIMMUNOLOGY OF STRESS

INVESTIGATOR: DANIEL JJ CARR, PH.D., PRINCIPAL INVESTIGATOR

REPORT DATE: AUGUST 20, 1995

TYPE OF REPORT: ANNUAL REPORT - SEPT. 21, 1994 THROUGH JULY, 1995

TABLE OF CONTENTS

OVERVIEW

This project is organized around the central hypothesis that stressors (temperature and restraint) have detrimental effects on immunocompetence potentially resulting in the reactivation of latent pathogens. Our working model is a latently-infected herpes simplex virus type-1 (HSV-1) mouse. Our central hypothesis is that the hypothalamic pituitary adrenal (HPA) axis and the sympathetic nervous system (primarily adrenergic pathways) are the parties responsible for HSV-1 reactivation. Alterations in serum corticosterone and shifts in monamines in the brains, trimgeminal ganglia, and brain stems are predicted to correlate to the reactivation status of the stressed mice. In addition, assessing the local immune parameters (specifically cytokine production) and relating their levels to viral reactivation following stress are predicted to be helpful in identifying and elucidating a(the) mechanism associated with viral reactivation. We hypothesize that corticosterone (following HPA activation) and local (trigeminal and sacral ganglia) increases in neurotransmitters (catecholamines) following stress modify the local immune profile leading to virus reaction ultimately resulting in viral shedding and pathogenesis. The pathogenesis can manifests as simple mucocutaneous lesions or potentially develop into more severe complications. Consequently, the degree of discomfort could in fact incapacitate personnel in the form of mental anguish and/or physical impairment or discomfort resulting in poor performance. It is anticipated that by identifying the primary mechanism of viral reactivation, pharmacotherapeutic strategies can be applied reducing or eliminating viral reactivation.

BUDGET OF THIS YEAR'S REPORT

SALARIES:	22,080
TRAVEL:	1,500
OPERATING SERVICES:	3,607
SUPPLIES:	10,908
EQUIPMENT:	3,000
SUBTOTAL:	41,095
INDIRECT COSTS:	6,400
TOTAL:	**47,495**

JUSTIFICATION OF THE BUDGET

SALARIES: 12% of the P.I.'s institutional salary ($47,187) and 100% of the graduate student's stipend ($14,000) plus fringe.

TRAVEL: The P.I. and graduate student presented at three national conferences (6th Psychoneuroimmunology Research Society Conference, Key Biscayne, FL; 55th Annual CPDD Conference, Scottsdale, AZ and 9th International Congress of Immunology, San Francisco, CA) studies that were supported by The Department of Defense.

OPERATING SERVICES: Supports the service contract (with Beckman Instruments) for the scintillation counter that is required for some of the work outlined in this project.

SUPPLIES: Supports the purchase of all reagents and animals as well as the per diem for the animals.

EQUIPMENT: No equipment has been purchased this year. This money is beng redistributed to supplies so that additional experiments can be carried out.

NOTE: This project was originally assigned a budget of $80,684. However, the original P.I. (Dr. Bryan Gebhardt) and the program project director (Dr. Nicolas Bazan) graciously agreed to divide this project in half. However, this decision does not reflect any disagreement or incapatability between parties. Rather, it allows Dr. Gebhardt (P.I. on project 6A) and Dr. Carr (P.I. on project 6B) to focus more closely on their expertise and goals of each specific aim. Moreover, Drs. Carr & Gebhardt interact on a weekly basis over the project, data collection, and analysis of data.

Due to the workload of this project, an undergraduate student (Ms Gina Schilleci) was hired ($5.00/hr) to assist the graduate student (Bill Halford) with the viral infections, plaque assay, virus titers, and tissue culture. Furthermore, due to the relatively small budget, Dr. Gebhardt incurred some of the expenses for animal husbandry on this part (6A) of the project.

PERSONNEL

DANIEL JJ CARR, PH.D.	PRINCIPAL INVESTIGATOR
WILLIAM P. HALFORD, M.S.	GRADUATE STUDENT
GINA SCHILLECI	UNDERGRADUATE STUDENT

Dr. Carr is responsible for the overall organization of this project. Dr. Carr oversees the progress of the graduate student and helps plan and interpret experiments and experimental designs.

Bill Halford is responsible for the all facets of the work associated with this project. Consequently, he infects and screens mice for HSV-1, processes the tissue, cultures latently-infected cells, carries out the RT-PCR for viral and cytokine transcripts, and packages the results for statistical analysis. This project will be the major part of Mr. Halford's dissertation.

Ginal Schilleci assists Mr. Halford in his every day duties as well as organizes and restocks supplies needed in the lab for this project.

ANIMAL USE
1 OCTOBER, 1994, THROUGH JULY 1995

DAMD17-93-V-3013

The experimental animals used during this period for the project, Neural Responses to Injury: Prevention, Protection, and Repair, subproject: **Neuroimmunology of Stress, Injury, and Infection**, are as follows:

Species	Number of animals allowed	Number used	LSU IACUC #
Mouse	200	160	1257

**It should be noted that additional mice (app. 240) were used under a IACUC protocol provided by Dr. Gebhardt (P.I. under chapter 6A).

OTHER SUPPORT

During this year, Dr. Carr was awarded two other small grants:

Ladies Leukemia League: "Molecular Studies on Transcriptional Factors: Association with Leukemia." May 15, 1994 - July 31, 1995. $20,959 total costs. The study investigated the levels of transcriptional factors along with cytokines in splenic lymphocytes obtained from TAT72-transgenic and non-transgenic mice.

LSU Neuroscience Center. "Assessment of the Immune Response to HSV-1 Reactivation in HSV-1 Latently-Infected Mice: In Vitro Correlate." April 3, 1995 - June 30, 1995. $10,000 total costs. The study was centered on developing an *in vitro* corollary for HSV-1 reactivation. The study complimented work progressing from the DoD-supported grant.

SPECIFIC AIMS

**Each original specific aim will be listed and progress on each will be presented along with the significance and future plans.

SPECIFIC AIM #1: **To determine the effects of a brief period of thermal stress (10 min at 43^{o}C) and restraint stress (60 min) as indirect mediators of HSV-1 reactivation from neural tissues.**

A. Determination of viral reactivation in co-culture. These experiments were designed to allow us to determine the frequency of viral reactivation following moderate stressful events and to establish the baseline from which future experiments could be planned. The results presented in the first year's progress report (Tables 1 & 2) showed that the heat stress paradigm induced a

higher percentage of reactivations (80% or 8/10 mice reactivated) as compared to the restraint stress model (40% or 4/10 mice reactivated) as determined by the recovery of infectious virus in the trigeminal ganglia, corneas, and tear film. This experiment was repeated three times with similar results each time. It was noted that statistical analysis of the data indicated a high degree of significance ($p<.005$) as determined by ANOVA. In mice that were not stressed, no recovery of infectious virus was found in any tissue examined. In both cases, the mice were ocularly infected followed by a 35 day incubation period.

B. Immunohistochemical detection of viral reactivation in neural tissue. No experiments have thus far been carried out to immunohistochemically define viral reactivation. However, in the upcoming year, studies will be initiated to section trigeminal ganglia 24-72 hr post heat stress in virally-infected mice and immunohistochemically define the presence of HSV-1 in the trigeminal ganglia using anti-HSV-1 antisera (DAKO).

C. Use of nucleic acid amplification (PCR) to detect stress-induced viral reactivation. We have attempted to use reverse transcriptase (RT)-polymerase chain reaction (PCR) to detect HSV-1 lytic phase transcripts of all three kinetic classes (i.e., immediate-early, delayed-early, and late) as a marker of HSV-1 reactivation following hypothermic stress of latently-infected mice. The HSV-1 specific primers chosen amplify infected cell polypeptide 27 mRNA (i.e., ICP27, immediate early), ribonucleotide reductase mRNA (i.e., RR, delayed-early), and virion protein 23 mRNA (i.e., MP23, late). The ICP27 and VP23 PCR primers have been used before to detect HSV-1 mRNAs during reactivation in a rabbit model (Bloom et al., 1994). However, we have found that aside from a single experiment where ICP27 mRNA was detected in 2/2 mice (115 days post infection) 12 hours after hyperthermic stress, detection of HSV-1 lytic phase transcripts following hyperthermic stress has been inconsistent (2/16 mice) (see Appendix, Fig. 1).

One notable observation that has arisen out of efforts to detect HSV-1 lytic phase transcripts as markers of reactivation is the detecton of VP23 mRNA in the TG of 16/16 latently-infected mice (i.e., 30-44 days post infection), regardless of stress treatment. The identity of these PCR products has been confirmed by Southern blotting (see Appendix, Fig. 2). The possibility that the VP23 PCR product was amplified from contaiminating HSV-1 DNA in the RNA preparation has not been formally ruled out. However, this is unlikely since screening the RT products generated from the mRNA for ICP27 did not detect product. The presence of a HSV-1 RNA species other than the latency associated transcript (LAT) is of interest because there are no known latency-associated proteins, and such a a protein may potentially play an important role in the latent phase of the HSV-1 life cycle.

Significance: The stressors chosen for use in these investigations are bonafide methods of inducing viral reactivation. We have subsequently chosen to employ the heat stress paradigm in all future experiements.

SPECIFIC AIM #2: **To determine the neuroendocrine mechanism of stress-induced viral reactivation by measuring corticosterone and monoamine levels in the serum and nervous tissues of latently infected, stressed animals.**

The goal of this specific aim was to initiate studies in quantitating tissue monoamine and serum corticosterone levels in the stressed mice (infected and uninfected) to determine if a correlation exists between those levels and viral reactivation. In the progress report for year 1 (last year), we presented data measuring serum corticosterone and brainstem monoamine levels 24-hr following hyperthermic stress. Even though this time point is not ideal for such measurements (since neurotransmitter and HPA axis hormone release are rapid responders to stress), the results show that the procedure in isolating and processing tissue allow us to make accurate measurements. We found no differences in the monoamine levels measured (norepinephrine, epinephrine, dopamine, and serotonin) in the brainstem. Serum corticosterone levels were elevated in the stressed mice compared to the non-stressed infected mice. However, there were no differences between serum corticosterone levels between stressed and non-stressed uninfected mice. Potentially, the virally-infected animals which are in a process of viral reactivation may have signalled immune cells to secrete ACTH which could then activate the HPA axis ultimately resulting in an elevation in corticosterone production. Precedence for the involvement of leukocytes in the HPA axis has previously been reported (Blalock, 1984).

Significance: Based on the preliminary studies, we feel confident that accurate measurment can be obtained using both HPLC and RIA to measure monoamines and corticosterone respectively. Consequently, future plans are to undertake a time course study sampling tissues (trigeminal ganglia and serum) from HSV-1 infected and non-infected mice that have or have not undergone hyperthermic stress. Time points that will be taken include 0 min, 30 min, 60 min, 6 hrs, and 12 hrs post-stress episode. Measurements for monoamines and corticosterone will be taken. It is anticipated that these results will be reported in next year's progress report. We currently would like to focus our effort under aims #3 and #4.

SPECIFIC AIM #3: **To determine the effect of stress on antiviral immune responses in mice undergoing viral reactivation.**

A. Serum antibody levels in control and stressed mice. Mice rendered latent for HSV-1 (we use 35 days post-infection) were planned on being sacrificed immediately after stress and at 4, 24, and 96 hr post stress. Antibodies for HSV-1 found in the serum were to be measured by ELISA as described (Gebhardt & Hill, 1988). Dr. Gebhardt (P.I., chapter 6A) has made significant headway in this area.

B. The effect of stress-induced viral reactivation on the CTL reactivity and interferon (IFN)-γ production in mice. In this aim, the splenic lymphocytes from mice sacrificed under Aim #3A will be assayed for CTL activity and IFN-γ production using ^{51}Cr-labeled fibroblasts. We have currently not performed any experiments to measure CTL activity nor IFN-γ production by splenic or lymph node lymphocytes. However, the lab has the capacity of measuring both and preliminary experiments are planned to undertake this investigation in the next fiscal cycle. Preliminary studies indicate that splenic lympphocytes obtained from HSV-1 latently-infected mice produce no measurable IFN-γ but rather IL-2, IL-6, & IL-10 compared to splenic lymphocytes from naive mice when stimulated with HSV-1-infected TGs *in vitro* (data not shown).

C. Cytokine expression in stressed mice during viral reactivation. The goal of this experiment is to investigate the effect of stress on the expression of cytokine genes in the neural tissues and spleens of HSV-1 latently-infected mice. The major amount of time and energy this past year has been devoted to this particular aim. In the experimental design, we investigated the levels of mRNA for proinflammatory cytokines (i.e., IL-1α, IL-6, and TNF-α). In addition, we have also investigated the mRNA levels for other cytokines including IFN-γ and IL-10 as well as the T cell-derived chemokine RANTES. The measurement of mRNA was via reverse transcription-polymerase chain reaction (RT-PCR) to order to insure a sensitive method of detection (more sensitive than a Northern for example). In these experiments, RT-PCR products were obtained from isolating RNA from the trigeminal ganglia of latently infected (INF) or uninfected (UI) mice at time points (t=) 0, 12-, and 24-hrs post hyperthermic stress.

The results shown in Figure 3 (see Appendix, Fig. 3) demonstrate the effects of HSV-1 infection and hyperthermic stress on the cytokine transcription profile within the trigeminal ganglia (TG; i.e. site of viral reactivation). Figure 3 shows two control amplifications which we perform on cDNA samples. Amplification for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA serves as a control for the quality of the cDNA preparation (Fig. 3a). Amplification for the HSV-1 latency-associated transcript (LAT) verifies that each mouse is latently infected (Fig. 3b). The RT-PCR samples shown in these two gel photographs, as well as the other six gel photographs in Figure 4, were amplified from TG cDNA of the same 9 latently infected mice and the same 8 uninfected mice.

Transcription of pro-inflammatory cytokine mRNAs in TG:
Effects of latent HSV-1 infection and hyperthermic stress

The results of RT-PCR comparison of pro-inflammatory cytokine mRNA transcription in the TG of latently infected and uninfected mice (see Appendix, Fig. 4) reveals some interesting differences. Thirty to forty days post-inoculation (PI), IL-1α mRNA expression in the TG is elevated in approximately one-third of latently infected mice, but in the other two-thirds is expressed at the same basal level as uninfected mice (Fig. 4a). While all TNF-α mRNA detected by RT-PCR in latently infected mouse TG is fully spliced, a significantly larger fraction of incompletely spliced TNF-α mRNA is found in uninfected TG (Fig. 4b). Given that TNF-α expression is largely regulated at the post-transcriptional level, this is a potentially significant finding whose relevance needs to be further explored. Hyperthermic stress appears to transiently upregulate IL-6 transcription in the TG, such that IL-6 mRNA levels are elevated at 12 h post stress, but have returned to basal levels by 24 h post stress (Fig. 4c). The hyperthermic stress-induced IL-6 mRNA profile in the TG appears similar in both infected and uninfected mice. The significance of this observation to HSV-1 reactivation is unclear at this point, but clearly demonstrates that the hyperthermic stress model we are using has measurable physiologic effects.

Transcription of T lymphocyte-associated cytokine mRNAs in TG:
Effects of latent HSV-1 infection and hyperthermic stress

RT-PCR comparison of T lymphocyte-associated cytokine mRNA transcription in latently infected TG and uninfected TG indicates that increased transcription of IFN-γ, IL-10, and

RANTES mRNA occurs during latent HSV-1 infection (Figure 4d-f). While there are clear differences between the TG of latently infected and uninfected mice, neither IFN-γ (Fig. 4d), IL-10 (Fig. 4e), nor RANTES (Fig. 4f) mRNA levels in the TG appear to be affected by hyperthermic stress. The infected group of mice whose RT-PCR profile is shown in Fig. 3 were sacrificed 30 days PI. Elevated levels of T cell-associated cytokine mRNA have also been observed in other experiments using mice sacrificed 39 days PI (Fig. 6), 44 days PI (Figure 8), and 115 days PI (data not shown).

These results strongly indicate an association between latent HSV-1 infection and elevated levels of IFN-γ, IL-10, and RANTES mRNAs in the TG. Therefore, it appears that increased numbers of T lymphocytes remain in the TG well after the resolution of acute HSV-1 infection. In order to more fully elucidate the differences in the levels of mRNA for the cytokines that have been tested and found to have some relevance to HSV-1 infection and potentially latency, we propose to use competitive RT-PCR to quantitate differences.

In order to accomplish competitive RT-PCR, we designed a series of mimetics as well as primers that could be used with the mimetics to generate cDNA that could effectively compete with our unknown cDNA templete (generated by RT from the extracted mRNA). We have successfully been able to test the competitive RT-PCR for IL-6, IL-10, and IFN-γ (see Appendix, Fig. 5). Accordingly, we are now in a position to quantitate these cytokine levels in the trigeminal ganglia of the infected and un-infected mice with or without stress.

Based on the hypothesis that infiltrating T lymphocytes are responsible for the observed increase in IFN-γ, IL-10, and RANTES mRNA in latently infected trigeminal ganglia (TG), we have made preliminary attempts at depleting T cells in latently infected mice in order to address the following two questions:

1) Can systemic T cell depletion be utilized to decrease the amount of IFN-γ, IL-10, and RANTES (and their respective mRNAs) in the TG of latently infected mice?
2) Will T cell depletion increase the susceptibility of latently infected mice to HSV-1 reactivation?

In our first preliminary experiment, latently infected mice received 3 subsequent daily i.p. injections of rabbit anti-mouse T cell polyclonal antiserum (i.e. samples D45 and 46) or phosphate-buffered saline (PBS; samples V45 and V46). RT-PCR comparison of TG cytokine profiles in the two treatment groups revealed no obvious differences in IFN-γ, IL-10, or RANTES mRNA expression (see Appendix, Fig. 6). FACS analysis of splenic lymphocytes confirmed that there was a depletion in $CD8^+$ lymphocytes (see Appendix, Fig. 7) in mice receiving anti-T cell antiserum relative to PBS-injected controls, however, no such decrease was observed in $CD4^+$ lymphocytes.

In a second T cell depletion experiment, latently infected mice treated with either anti-T cell antiserum (i.e. sample D47) or PBS (i.e. samples V47 and V48) were hyperthermically stressed, and sacrificed 24 hours after the stressor. Levels of IFN-γ, IL-10, and RANTES mRNA appeared to be lower in the antiserum-injected/ heat-stressed mouse relative to PBS-injected / heat-stressed controls (see Appendix, Fig. 8). While the results are encouraging, more work is required.

Significance: Based on these preliminary results reported above and reproduced again for an n value of 5/group tested, we feel confident that further exploration in determining the levels of some of these cytokines will bear fruition to the relationship between cytokine levels and viral reactivation. To this end, we plan on undertaking competitive RT-PCR for the quantitative determination of mRNA extracted from the trigeminal ganglia at different time points (4-24 hrs) (of HSV-1 infected and un-infected mice with or without stress) encoding IL-6, IL-10, IFN-γ, and the T cell-derived chemokine, RANTES. In addition, protein levels for these cytokines in the trigeminal ganglia will also be determined by ELISA (IL-6, IL-10, and IFN-γ are routinely measured in this lab) or immunohistochemically (RANTES). The results of these experiments will be used along with a series of planned experiments using an *in vitro* HSV-1 reactivation system to fully elucidate the role of cytokines in HSV-1 reactivation or hindrance of reactivation following stress.

Relative to defining T cell subsets that may be involved in the regulatory effects of HSV-1 reactivation following stress, work is planned to deplete initially all T cells ($CD3^+$, $CD4^+$, and $CD8^+$) and subsequently deplete selective subpopulations to determine if one or more of the populations is responsible for the cytokine mRNA expression in the TG.

SPECIFIC AIM #4: **To determine the effect of exogenous stress hormone agonists and antagonists on stress-induced viral and antiviral immunity.**

The hypothesis on which this investigation is based centers on the notion that corticosterone and/or catecholamines are in part responsible for the reactivation of HSV-1 in latently infected mice following hyperthermic stress. Accordingly, the administration of exogenous catecholamines or corticosteroids are predicted to reactivate HSV-1 without subjecting the latently infected animals to the stress paradigm.

Progress has been made in this aim using both *in vivo* and *in vitro* systems. Specifically, the administration of propranolol (β-adrenoceptor antagonist) has been shown to suppress the reactivation of HSV-1 latently-infected mice (Gebhardt & Kaufman, in press, see chapter 6A). These results would suggest that catecholamines may play a significant role in HSV-1 reactivation. Of course, future experiments will be required to show a dose-dependent effect as well as to differentiate between β_1 and β_2 mediated pathways using selective agonists and antagonists. However, this is the first reported case showing a direct cause/effect with adrenergic antagonists.

Likewise, our lab has utilized a dissociated TG culture paradigm of HSV-1 reactivation described by Moriya, et al. (1994) to test biological mediators for their ability to induce HSV-1 reactivation from latently-infected neurons. We have demonstrated that latent HSV-1 infection is maintained in these TG cultures by 1) detecting LAT mRNA in 10/10 dissociated TG monolayers ten days after plating (data not shown), and 2) by inducing efficient reactivation from these cultures with a hyperthermic stressor delivered 15 days after plating ranging from 70-90% reactivation of latently-infected neural cultures.

Since it was apparent the *in vitro* culture system paralled the *in vivo* hypothermic stress mouse model reactivation of latent HSV-1, a series of experiments were carried out to determine the

direct role of hormones and predicted association with reactivation of latent HSV-1 in the TGs. Latent HSV-1 did not reactivate from this dissociated TG culture system in response to any of the following drug treatments (n=8-14 wells/drug repeated at least twice): 10^{-6} M, 10^{-8} M, 10^{-10} M epinephrine; 50 μM and .5 μM forskolin, 500 μM chlorophenylthio-cAMP, and 10^{-7} M morphine (data not shown). The presence of latent, reactivatable HSV-1 in these TG cultures was confirmed after drug treatment by heat shock-induced reactivation (70-90 % reactivated). Therefore, while direct roles for epinephrine and cAMP have been postulated in stress-induced HSV-1 reactivation (Hill, et al., 1987; Smith et al., 1992), we find that neither of these mediators by themselves induce HSV-1 reactivation from dissociated, latently infected TG cultures.

As a correlate of our *in vivo* studies, we have attempted to measure the effect of hyperthermic stress (i.e. same treatment used to reactivate latent HSV-1 in this model) on cytokine production from both latently infected and uninfected dissociated TG cultures. We have found no detectable amounts of IL-10 (less than 50 pg/ml) IFN-γ, or TNF-α concentrations as determined by ELISA (with a sensitivity range down to 50 pg/ml for IL-10 and TNF-α and 30 Units/ml for IFN-γ). However, IL-6 is readily detectable in latently infected and uninfected TG cultures. We have found that 24 hours after heat stress, latently-infected TG cultures contain modestly elevated IL-6 concentrations while uninfected TG cultures contain significantly less IL-6 (Table I). Notably, our *in vivo* RT-PCR results suggest that a similar pattern of IL-6 induction occurs in latently infected mice following hyperthermic stress (Fig. 4c).

TABLE 1. HYPERTHERMIC STESS CHANGES IL-6 PRODUCTION BY TG CULTURED NEURONS[a]

HSV-1 INFECTED	Prior to hyperthermic stress	24-HR post hyperthermic stress	48-HR post hyperthermic stress	72-HR post hyperthermic stress
-	63.5 +/- 6.7[b]	37.7 +/- 5.3*	50.9 +/- 6.8	28.6 +/- 3.5**
+	64.0 +/- 7.9	80.2 +/- 7.6	52.5 +/- 4.4	41.6 +/- 4.6*

[a]TG neuron cultures were initiated from surgically removing the TGs from latently-infected ICR mice and processing the neurons as described (Moriya et al., 1994). HSV-1 reactivation has been monitored in each processed TG preparation (typically 10 TGs per 24-well microtiter plate). Typically, the processed TGs are placed in culture with or without (positive control) (E)-5-(2-bromovinyl)-2-deoxy-uridine (5-BVDU5 μg/ml). The concentration of 5-BVDU used in the cultures was emperically determined in our lab using HSV-1-latently infected TGs testing concentrations ranging from 0.5-500 μg/ml of 5-BVDU. After 10-12 days in culture, the HSV-1 latently-infected TG supernates were sampled and TG cultures were subsequently stressed (43° C for 180 min). Supernate samples were collected at 24, 48, and 72 hrs post stress and measured for IL-6.

[b]Numbers (ng/ml) are the mean +/- SEM, n=24/group as determined by ELISA. This table is the summary of 3 independent experiments using 8 wells per-infected or uninfected TG groupings/experiment. **$p<.01$, *$p<.05$ comparing the IL-6 levels post stress to pre-stress levels in both infected and uninfected TG cultures. There was no detectable IL-2, IL-10, or IFN-γ in any of the culture supernates tested (data not shown).

In parallel to the above mentioned studies investigating stress-induced reactivation of HSV-1 latently infected mice, the laboratory has been involved in investigating chemical stressors that might be involved in the pathogenic processes of HSV-1 infection or reactivation as well. Studies conducted by Holaday et al. while working in the Division of Neuropsychiatry of Walter Reed Army Institute provided evidence that morphine is a potent immunosuppresssive drug when

administered *in vivo*. The immunosuppression observed following morphine adminisntration suggested that T cell-mediated events including lymphocyte proliferation can be severely afftected following morphine exposure (Bryant et al., 1988; Bryant et al., 1991). Based on these observations, a series of studies were carried out to explore the effects of morphine on CTL activity (since these effector cells are central to anti-viral immunity). Four published manuscripts describe various experiments that show morphine suppresses CTL activity through the involvement of opioid receptors classified most closely with μ (see Appendix, manuscipts 1-4). A fifth article reviews the current working model developed in part in this laboratory by which morphine is predicted to suppress CTL activity (see Appendix, manuscript #5). The sixth article describes work in which the P.I. and graduate student have identified the first functional role for an orphan opioid receptor in the immune system (see Appendix, Manuscript #6).

It is anticipated that this work will be directly applicable to the goals of this proposal. Specifically, one of the studies (Carpenter et al., 1994) has shown that morphine acts as a co-factor in potentiating viral-induced encephalomyelitis in HSV-1 acutely-infected mice. Our initial experiments outlined under Aim #4 have suggested that morphine (10^{-7} M) does not alone reactivate latently-infected TGs *in vitro*. These results will be repeated at different doses of morphine (10^{-5} - 10^{-11} M). In addition, morphine will also be used to determine if it acts as a co-factor in hyperthermic stress induction of HSV-1 reactivation similar to what we have found in preliminary results using dexamethasone (10^{-7} - 10^{-11} M) (data not shown). Based on our hypothesis that morphine acts as a co-factor to HSV-1 reactivation as well as pathology (due to its immunosuppressive properties) associated with the acute infection, experiments are planned to determine if morphine alone can reactivate HSV-1 latently-infected mice or synergize with hyperthermic stress in the reactivation of HSV-1 latently-infected mice. The results of these experiments are in line to the mission of the Department of Defense since acute and chronic pain are typically treated using pain relief compounds including opioids. Since Bryant et al (1988) have shown morphine induced the activation of the hypothalamic pituitary adrenal axis production of corticosterone and we have preliminary data indicating the supercorticosterone, dexamethasone potentiates HSV-1 reactivation of latently-infected TGs, we hypothesize that a dynamic relationship will be elucidated between morphine and HSV-1 pathology and reactivation following hyperthermic stress.

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PUBLICATIONS RESULTING FROM THIS RESEARCH:

Publications:

1. Carpenter, G.W., H.H. Garza jr., B.M. Gebhardt, & D.J.J. Carr. 1994. Chronic morphine treatment suppresses CTL-mediated cytolysis, granulation, and cAMP responses to alloantigen. *Brain, Behavior, Immun.* 8:185-203.

2. Carpenter, G.W. & D.J.J. Carr. 1995. Pretreatment with β-funaltrexamine blocks morphine-mediated suppression of CTL activity in alloimmunized mice. *Immunopharmacol.* 29:129-140.

3. Carr, D.J.J. & G.W. Carpenter. 1995. Morphine-induced suppression of cytotoxic T lymphocyte activity in alloimmunized mice is not mediated through a naltrindole-sensitive delta opioid receptor. *Neuroimmunomodulation.* 2:44-53.

4. Carr, D.J.J., G.W. Carpenter, H.H. Garza jr., M.L. Baker, & B.M. Gebhardt. 1995. Cellular mechanisms involved in morphine-mediated suppression of CTL activity. *Adv. Exp. Med. Biol.* 373:131-139.

5. Halford, W.P., B.M. Gebhardt, and D.J.J. Carr. 1995. Functional role and sequence analysis of a lymphocyte orphan opioid receptor. *J. Neuroimmunol.* 59:91-101.

6. Halford, W.P., D.J.J. Carr. 1995. Subversion of intracellular signal transduction by herpes simplex virus type 1. *Adv. Neuroimmunol.* (in press)

7. Carpenter, G.W., L. Breeden, & D.J.J. Carr. 1995. Acute exposure to morphine suppresses CTL activity. *Int. J. Immunopharmacol.* (in press).

Abstracts:

1. Halford, W.P., B. Gebhardt, and D.J.J. Carr. 1994. HSV-1 latently infected mice display an altered response to stress: Implications for antiviral immunity. 6th Psychoneuroimmunology Research Conference, Nov. 17-20, Key Biscayne, FL

2. Halford, W.P., B.M. Gebhardt, and D.J.J. Carr. 1994. Mouse lymphocytes express an orphan opioid receptor. 6th Psychoneuroimmunology Research Conference, Nov. 17-20, Key Biscayne, FL.

3. Carr, D.J.J., L. Breeden, G.W. Carpenter, & B.M. Gebhardt. 1994. The frequency of exposure to morphine differentially affects CTL activity in alloimmunized mice. 6th Psychoneuroimmunology Research Conference, Nov. 17-20, Key Biscayne, FL.

4. Baker, M.L., D.J.J. Carr, & B.M. Gebhardt. 1994. Morphine suppresses peritoneal and splenic CTL activity in a dose dependent fashion in alloimmunized mice. 6th Psychoneuroimmunology Research Conference, Nov. 17-20, Key Biscayne, FL.

5.. Halford, W.P., M. Serou, B.M. Gebhardt, & D.J.J. Carr. 1995. Functional role and sequence analysis of a lymphocyte orphan opioid receptor. CPDD Meeting, June 10-15, Scottsdale, AZ

6. Halford, W.P., B.M. Gebhardt, and D.J.J. Carr. 1995. Analysis of the immune response during stress-induced reactivation of herpes simplex virus type 1. 9th International Congress of Immunology, July 23-29, San Francisco, CA.

APPENDIX

Item	Content
1.	Figures 1-8
2.	Carpenter et. al., 1994
3.	Carpenter & Carr, 1995
4.	Carr & Carpenter, 1995
5.	Carpenter et al., in press
6.	Carr et. al., 1995
7.	Halford et al., 1995

FIG. 1.

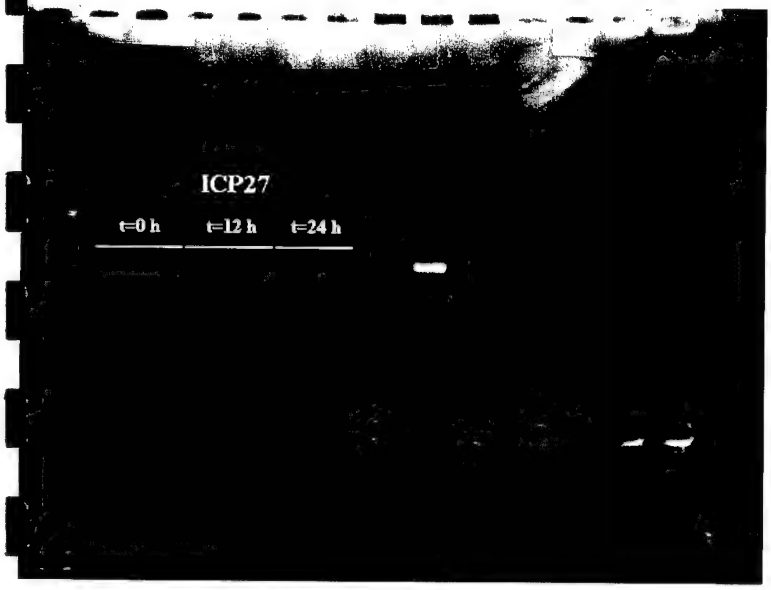


Fig 2

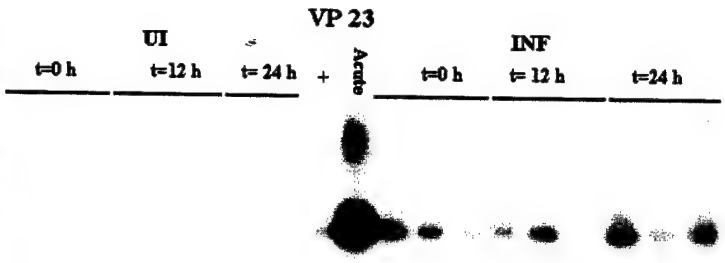


Fig 3a

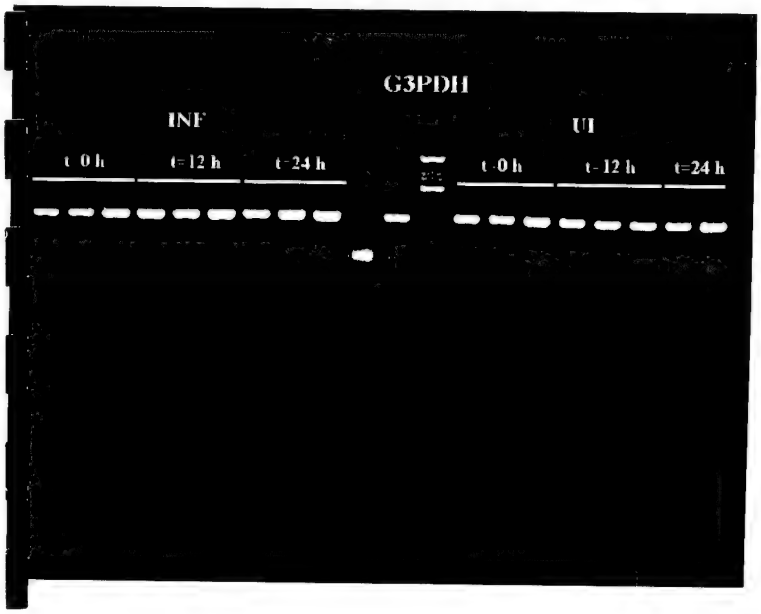


Fig 3b

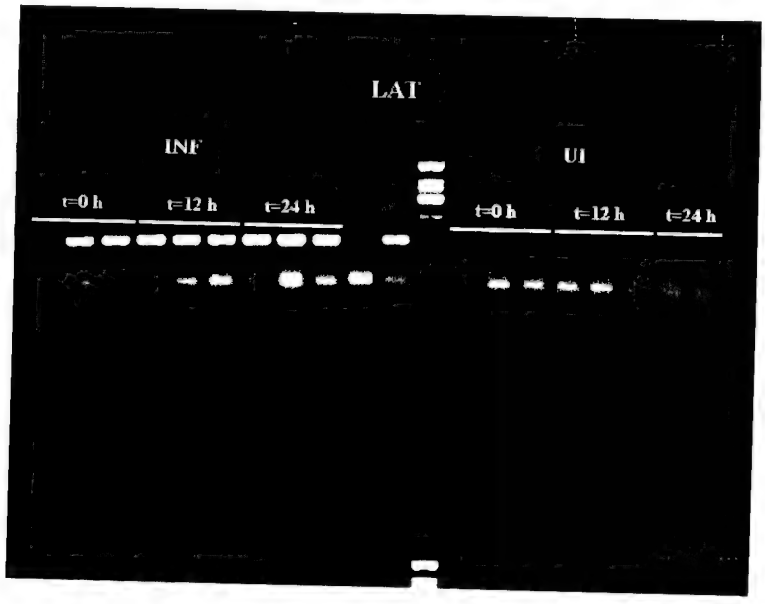


Fig 4a

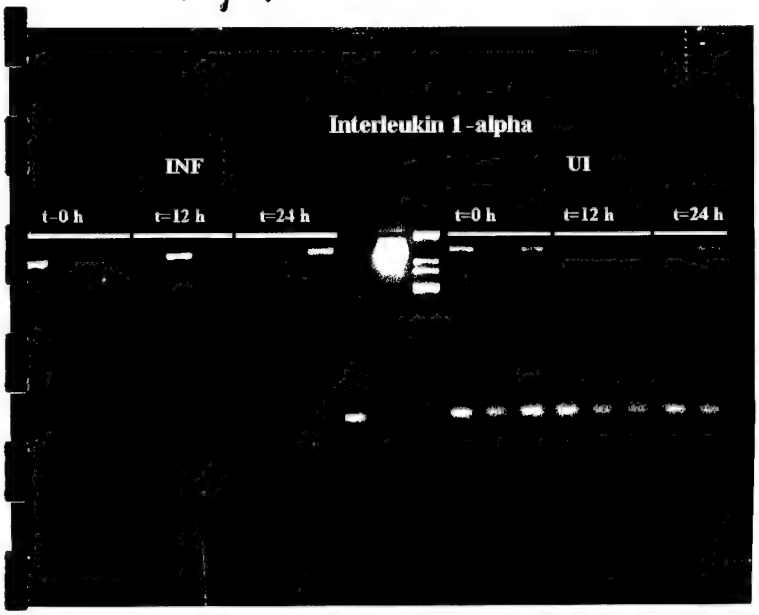
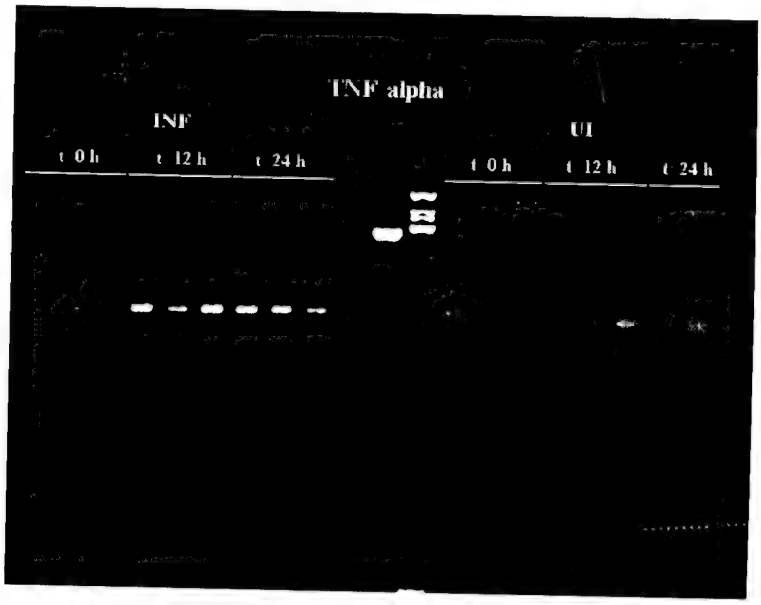


Fig 4b



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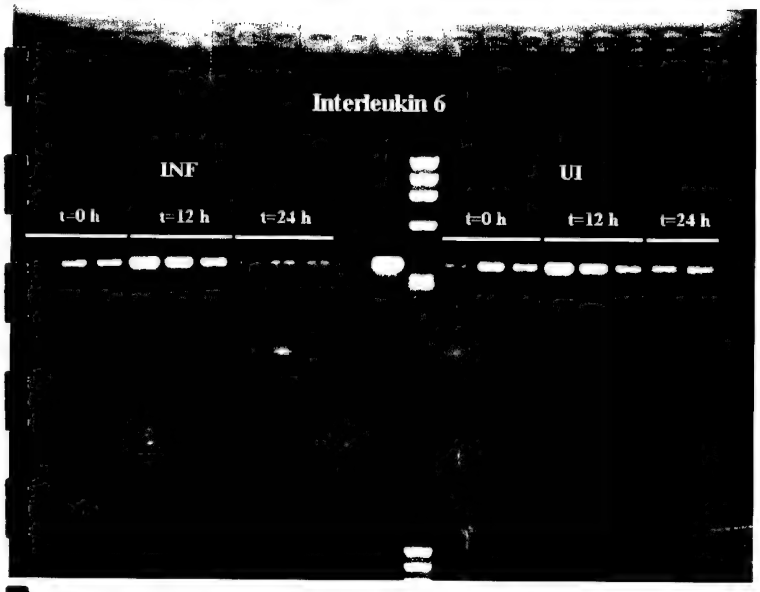

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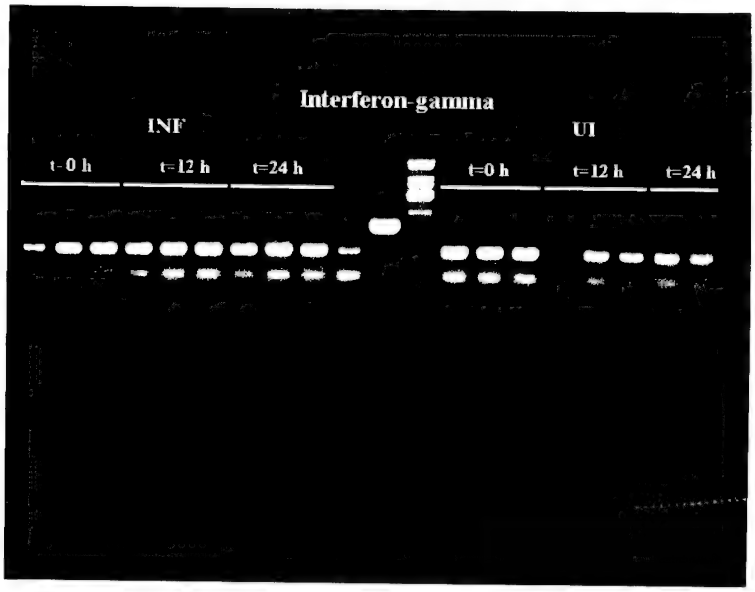

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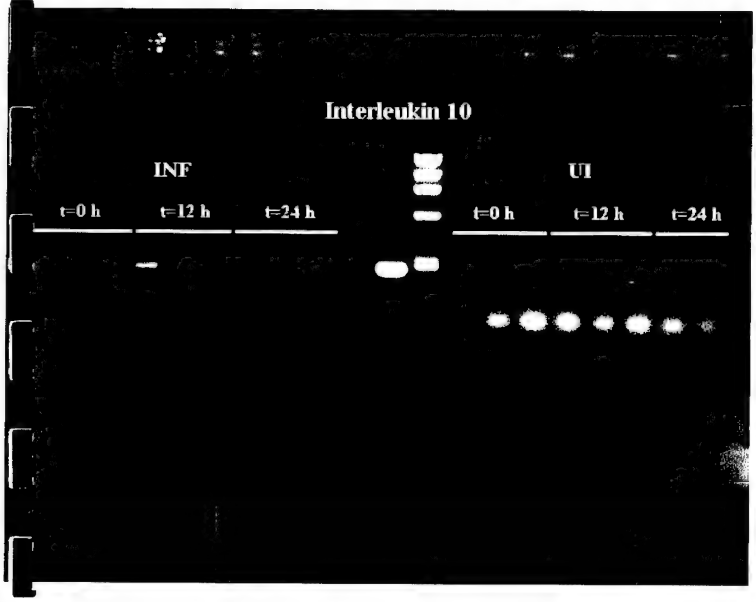
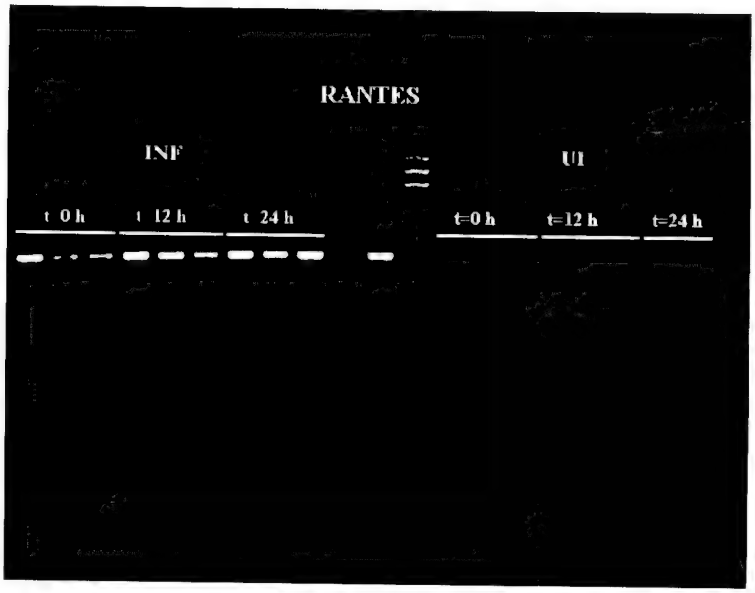


Fig 4f



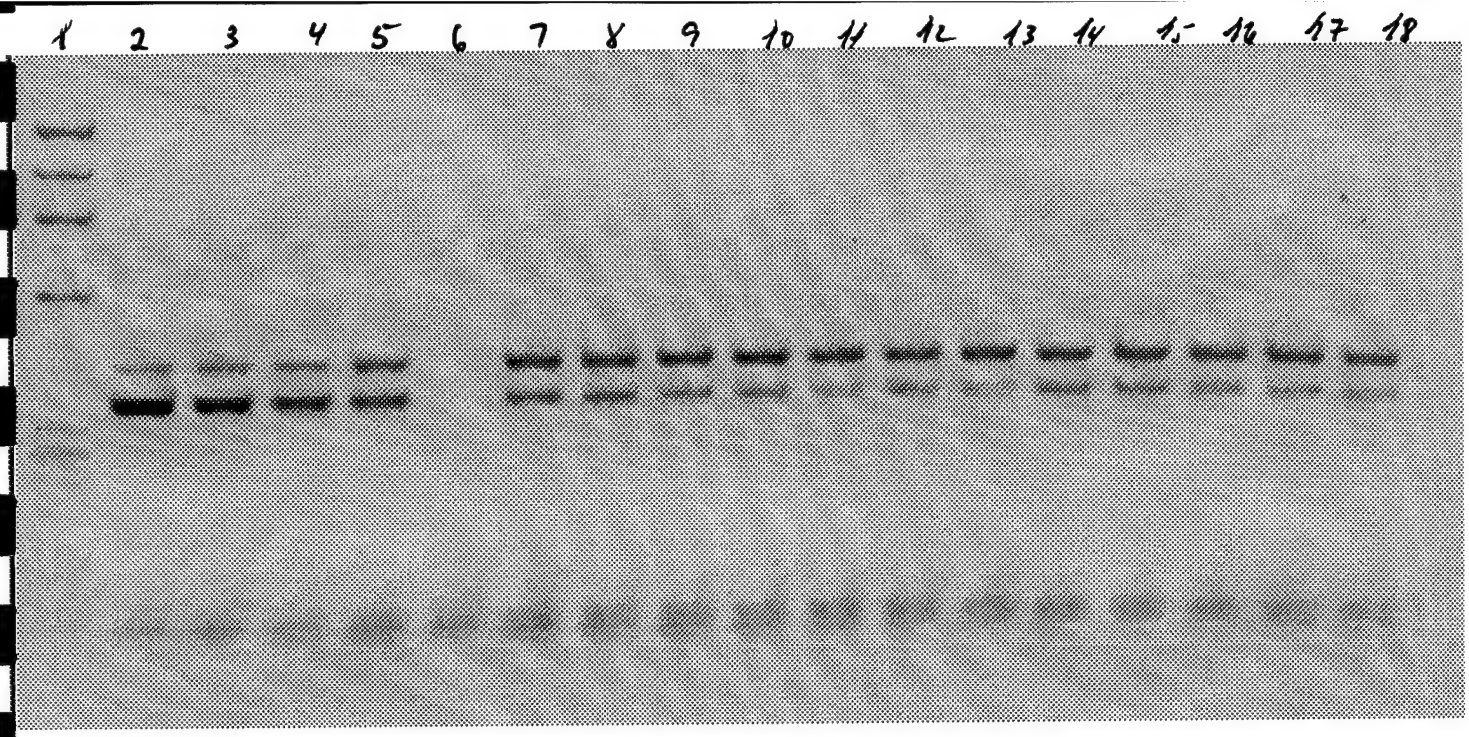


FIGURE 5A. RT-PCR of IL-6 transcript levels obtained from lipopolysaccharide-activated splenic lymphocytes. Total RNA was isolated from 10-40X10^6 splenic lymphocytes stimulated with 50 μg of lipopolysaccharide using Ultraspec (biotecx, Houston, TX). First strand cDNA synthesis was performed on 1 μg aliquots of total RNA using a cDNA synthesis kit purchased from Promega (Madison, WI). RT reactions were carried out at 42^o C for 1 hr using the poly(dT) primers provided. Once synthesized, specific cDNAs encoding IL-6 or glyceraldehyde-3-phosphate dehydrogenase were measured using primer competition PCR reactions. A series of PCR reactions were performed on aliquots of cDNA, each spiked with a known amount (12.5-800 copyequivalents) of PCR mimetic in order to create an internal standard curve (lanes 2-5). The PCR mimetic was a larger, internally non-homologous DNA fragment that competed for the same primers that amplified the target cDNA. After PCR amplification, the two species could be separated by agarose gel electrophoresis and analyzed densitometrically. When the cDNA aliquot was amplified to the same intensity as the internal competitive standard, they are termed "copy equivalent." Imagequant analysis can subsequently be used to quantitate the amount of copy equivalents per RT-PCR reactions and thus, per sample. A representative figure for IL-6 is shown above. Lane 1 is the DNA ladder; Lane 6 is the primer controls, and Lanes 7-18 are experimental samples.

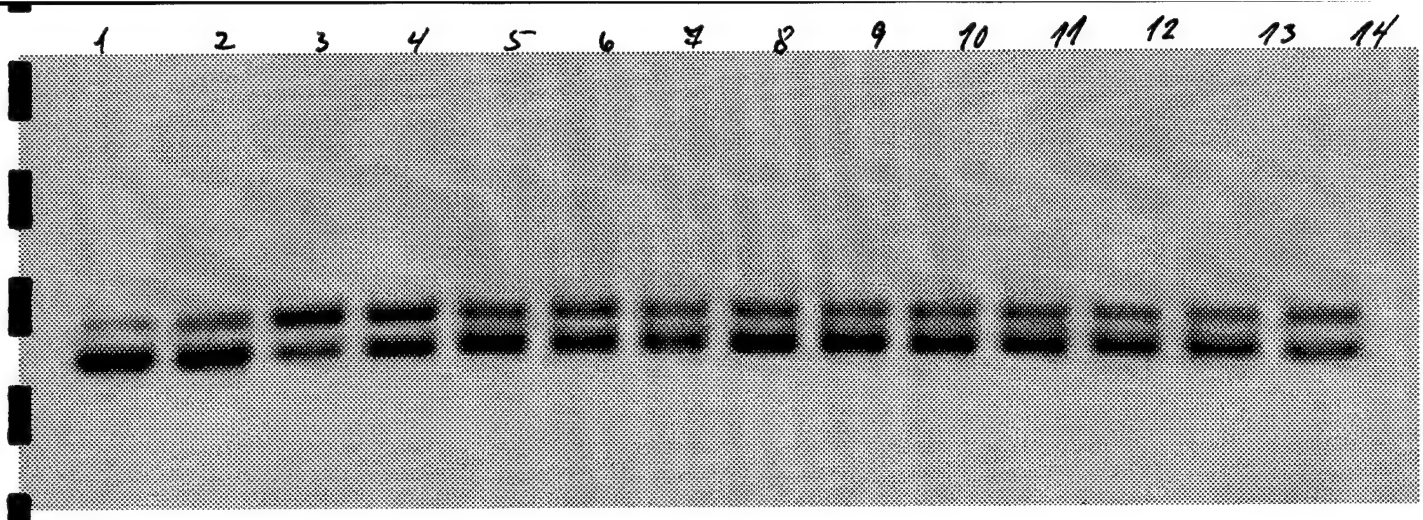


FIGURE 5B. RT-PCR of IL-10 transcript levels obtained from concanavalin A-activated splenic lymphocytes. Total RNA was isolated from 10-40X10^6 splenic lymphocytes stimulated with 75 μg of concanavalin A using Ultraspec (biotecx, Houston, TX). The RT-PCR set-up is identical as indicated in Fig. 5A legend. A representative figure for IL-10 is shown above. Lane 1-4 are the internal standard curve for the cDNA and mimetic of known copy equivalent 800-12.5 copy equivalents. Lanes 5-14 are experimental samples.

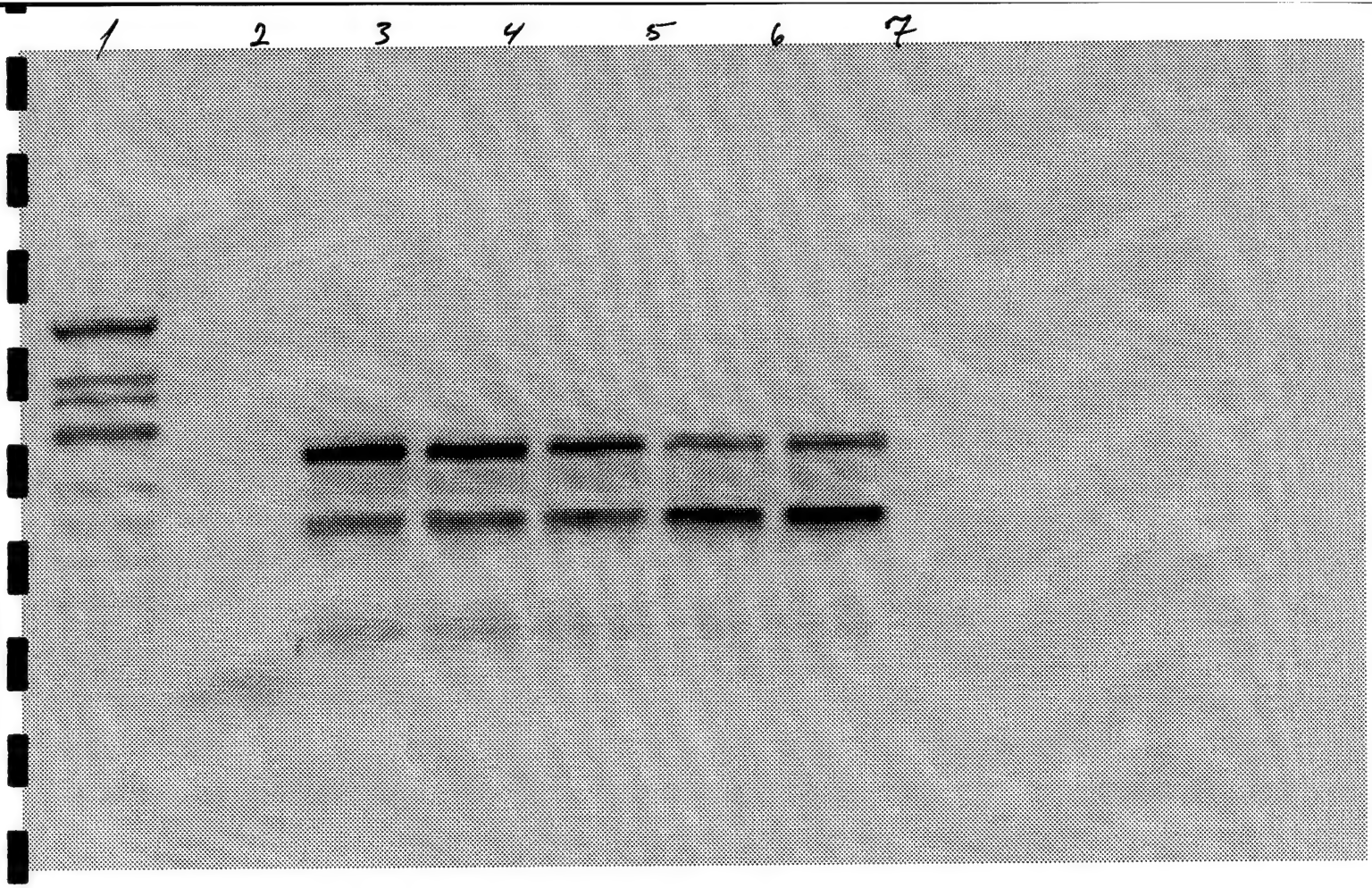
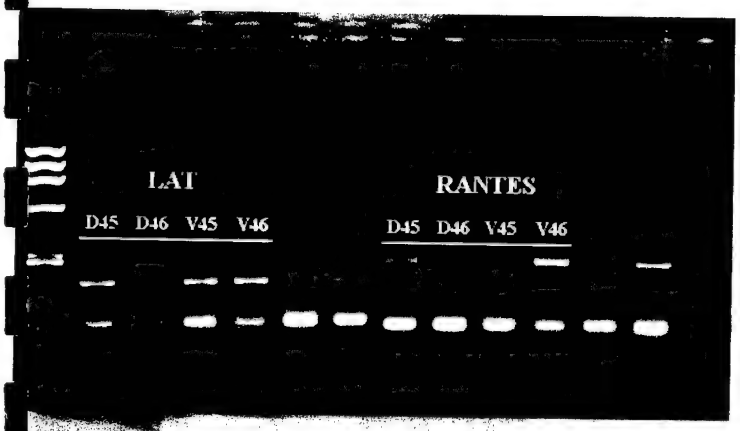


FIGURE 5C. RT-PCR of IFN-γ transcript levels obtained from concanavalin A-activated splenic lymphocytes. Total RNA was isolated from 10-40X10^6 splenic lymphocytes stimulated with 75 μg of concanavalin A using Ultraspec (biotecx, Houston, TX). The RT-PCR set-up is identical as indicated in Fig. 5A legend. A representative figure for IFN-γ is shown above. Lane 1 is the DNA ladder. Lane 2 is the primer control. Lanes 3-7 are the internal standard curve for the cDNA and mimetic of known copy equivalent 800-12.5 copy equivalents. No experimental samples are shown although they have been carried out. In addition, no competitive RT-PCRs are shown for the housekeeping gene G3-PDH although they have been carried out as well. Currently, two manuscripts using this technique and measuring cytokines (IL-2, IL-6, and IL-10) are in preparation for submission by Sept. 1, 1995.

Fig 6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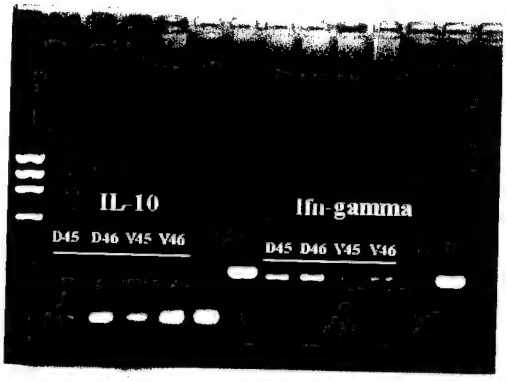
D45 D46 V45 V46
RANTES
D45 D46 V45 V46


IL-10
D45 D46 V45 V46
Ifn-gamma
D45 D46 V45 V46

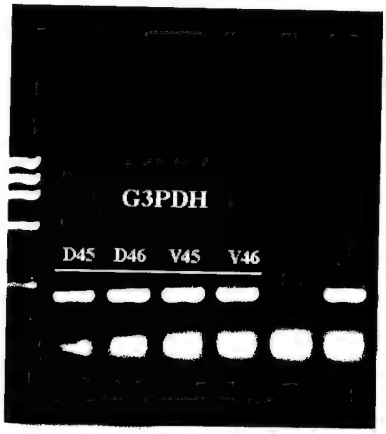
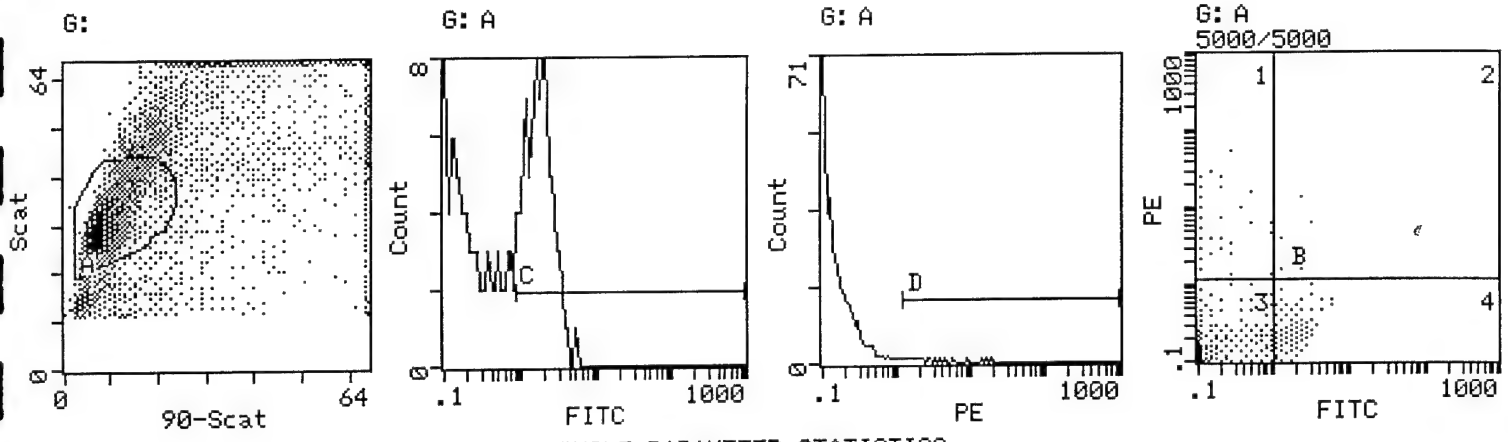

G3PDH
D45 D46 V45 V46

FIGURE 7. FACS ANALYSIS OF ANTI-T CELL TREATED MICE

FITC = CD4+
PE = CD8+

E. FACS PROFILE OF ANTI-T SERUM (D45)-TREATED MOUSE



SINGLE PARAMETER STATISTICS

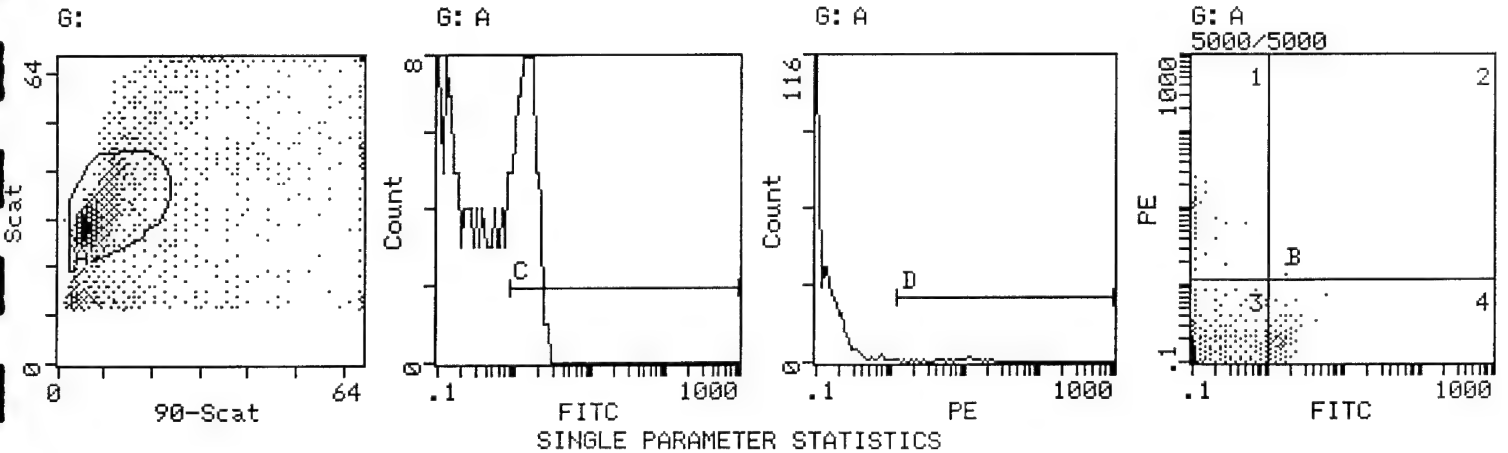
ID	Pcnt	Area	Peak Position	Peak Height	X Channel Mean	SD	FullCV	HalfCV	Min	Max
C	18.4	920	1.9	13	1.77	0.951	53.8	4.13	0.83	1024
D	3.7	186	1.6	4	4.89	5.77	118.0	0.446	1.2	1024

DUAL PARAMETER STATISTICS

ID	Pcnt	Area	Peak Position	Peak Height	X Channel Mean	X Channel SD	X Channel CV	Y Channel Mean	Y Channel SD	Y Channel CV
A	41.2	5000	7, 30	87	10.2	4.3	42.5	31.5	5.9	18.6

FITC = CD4+
PE = CD8+

F. FACS PROFILE OF ANTI-T SERUM (D46)-TREATED MOUSE



SINGLE PARAMETER STATISTICS

ID	Pcnt	Area	Peak Position	Peak Height	X Channel Mean	SD	FullCV	HalfCV	Min	Max
C	15.4	769	1.3	15	1.41	0.538	38.1	2.81	0.83	1024
D	5.3	266	9.0	6	6.66	6.75	101.4	0.420	1.2	1024

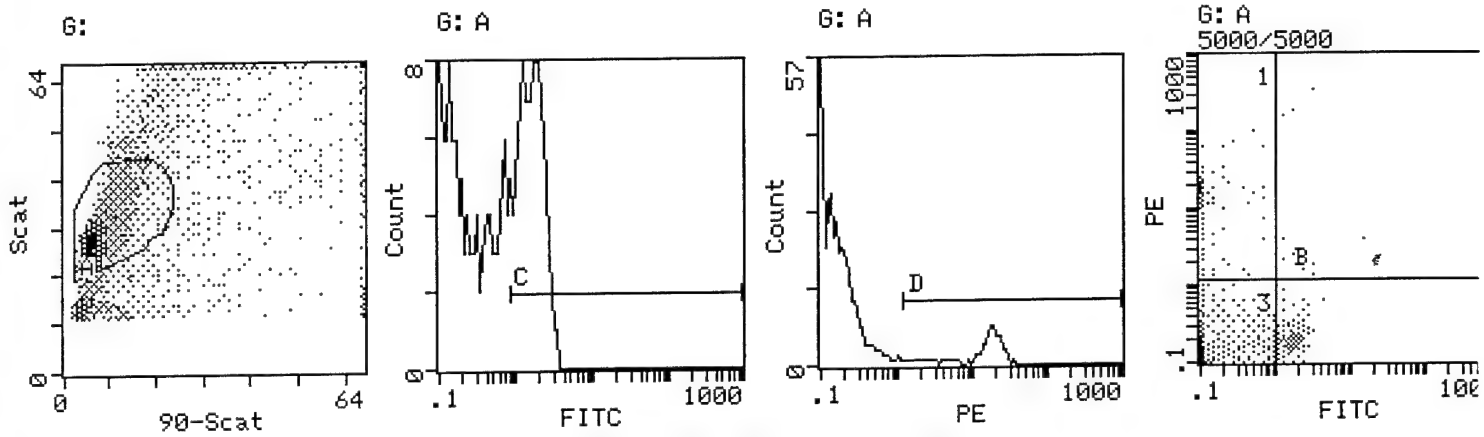
DUAL PARAMETER STATISTICS

ID	Pcnt	Area	Peak Position	Peak Height	X Channel Mean	X Channel SD	X Channel CV	Y Channel Mean	Y Channel SD	Y Channel CV
A	53.7	5000	5, 28	173	8.3	4.0	48.6	29.9	5.1	17.2

FIGURE 7. FACS ANALYSIS OF ANTI-T CELL TREATED MICE

FITC = CD4+
PE = CD8+

C. FACS PROFILE OF VEHICLE (V45)-TREATED MOUSE



SINGLE PARAMETER STATISTICS

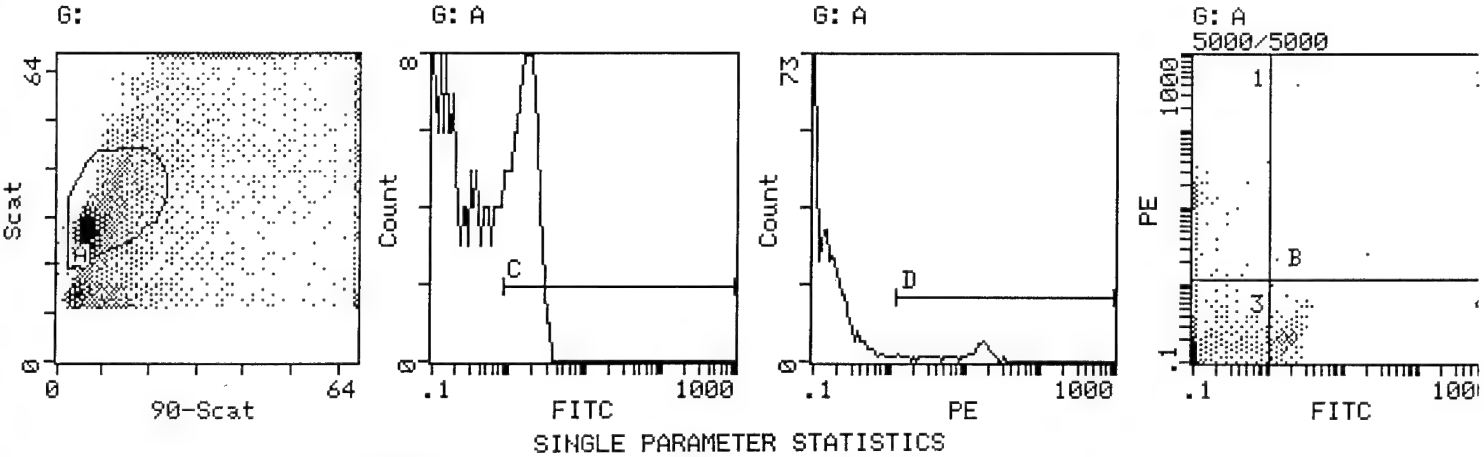
ID	Pcnt	Area	Peak Position	Peak Height	X Channel Mean	SD	FullCV	HalfCV	Min	Max
C	19.4	969	1.9	18	1.63	0.666	40.8	0.726	0.83	1024
D	13.6	681	17	13	11.5	11.1	96.2	0.559	1.2	1024

DUAL PARAMETER STATISTICS

ID	Pcnt	Area	Peak Position	Peak Height	X Channel Mean	X Channel SD	X Channel CV	Y Channel Mean	Y Channel SD	Y Channel CV
A	51.4	5000	5, 26	136	8.8	4.1	46.4	29.5	5.8	19.6

FITC = CD4+
PE = CD8+

D. FACS PROFILE OF VEHICLE (V46)-TREATED MOUSE



SINGLE PARAMETER STATISTICS

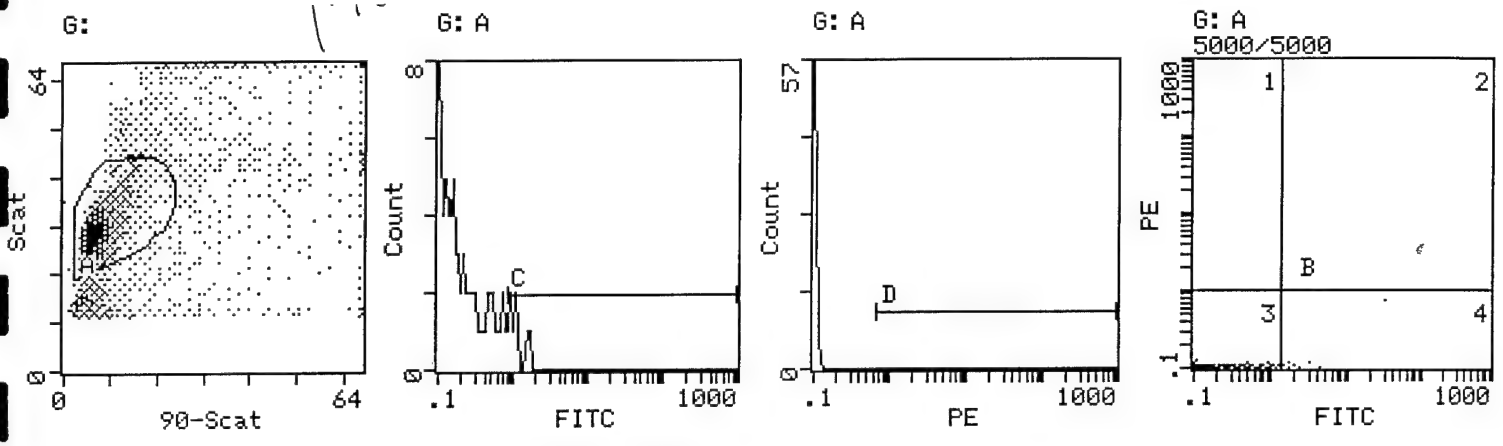
ID	Pcnt	Area	Peak Position	Peak Height	X Channel Mean	SD	FullCV	HalfCV	Min	Max
C	18.2	912	1.5	17	1.62	0.677	41.7	0.913	0.83	1024
D	10.1	504	16	9	9.47	9.84	103.9	0.630	1.2	1024

DUAL PARAMETER STATISTICS

ID	Pcnt	Area	Peak Position	Peak Height	X Channel Mean	X Channel SD	X Channel CV	Y Channel Mean	Y Channel SD	Y Channel CV
A	42.7	5000	5, 26	88	9.6	4.5	46.6	29.4	5.6	19.2

FIGURE 7. FACS ANALYSIS OF ANTI-T CELL TREATED MICE

A. ISOTYPIC CONTROL FLUORESCEIN ISOTHIOCYANATE (FITC)



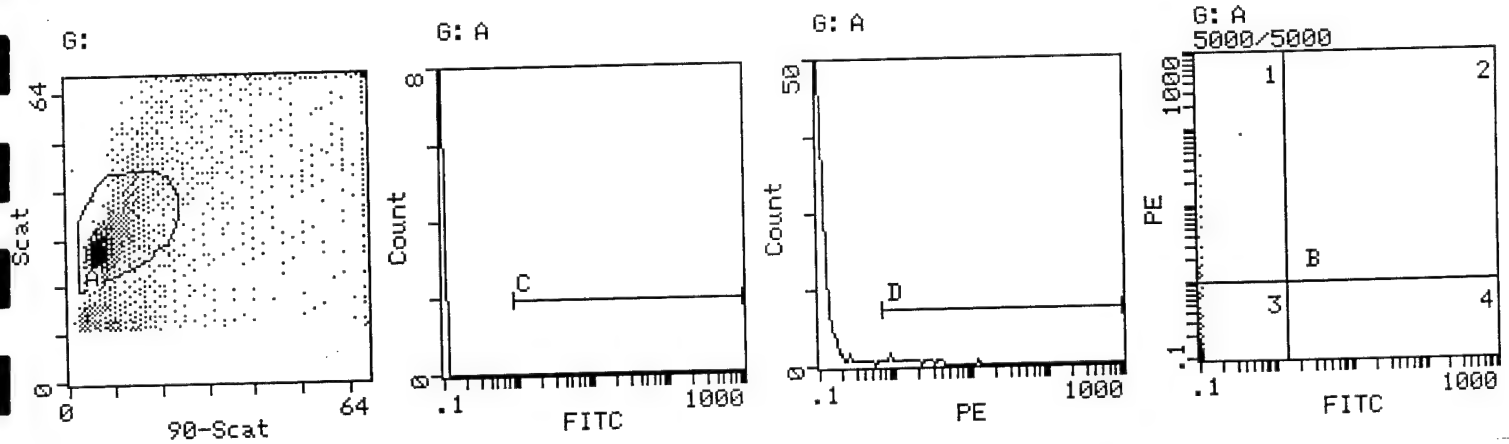
SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	2.3	113	0.90	6	1.51	1.14	75.8	0.573	0.83	1024
D	0.0	2	0.80	1	0.836	0.034	4.05	0.382	0.70	1024

DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	54.0	5000	5, 28	144	8.3	3.9	46.3	29.7	5.0	16.7

B. ISOTYPIC CONTROL PHYCOERYTHRIN (PE)



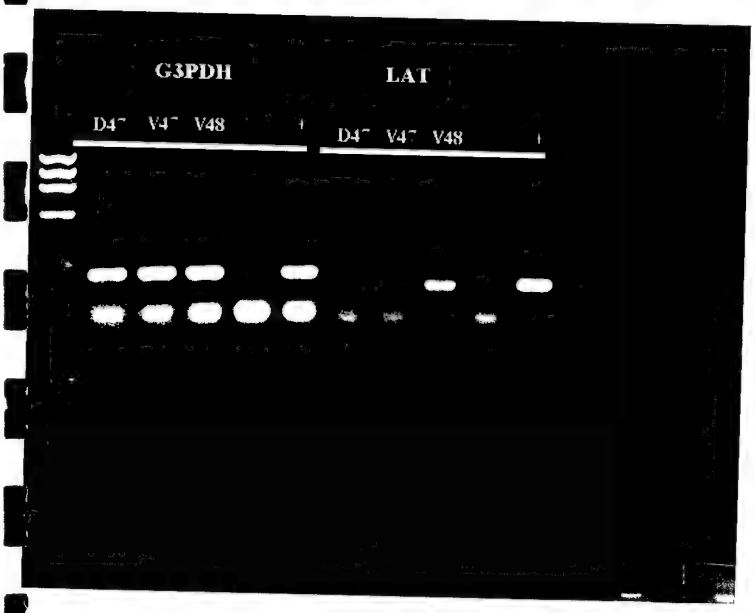
SINGLE PARAMETER STATISTICS

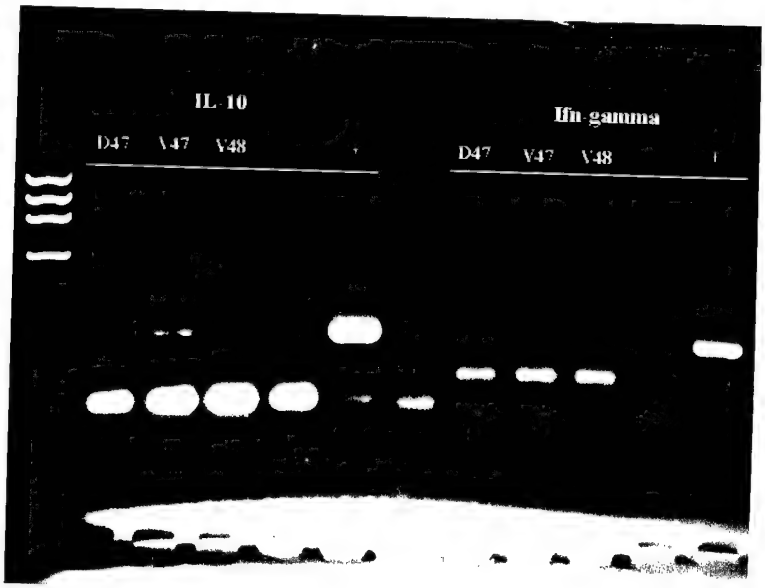
			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	0.1	4	1.2	1	3.35	3.45	102.9	0.382	0.83	1024
D	4.6	230	0.86	4	2.56	3.32	129.8	0.573	0.70	1024

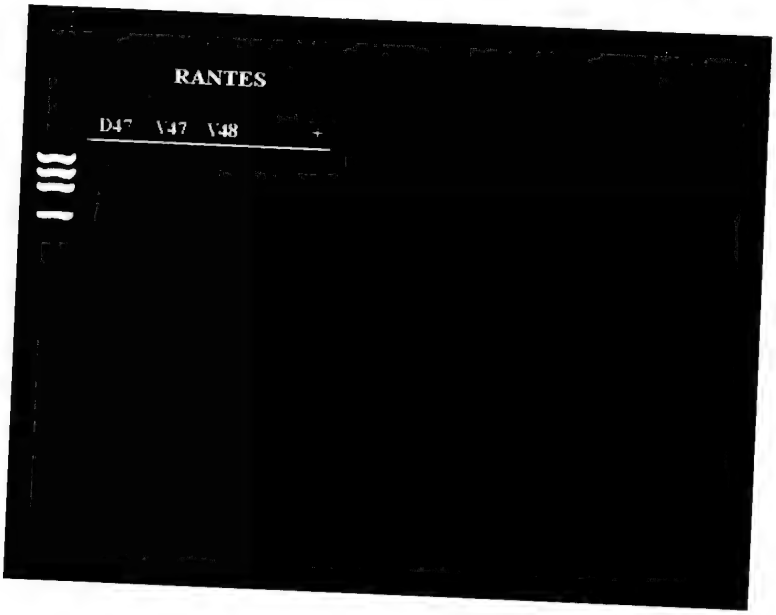
DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	49.6	5000	5, 26	108	9.1	4.3	46.8	29.8	5.5	18.3

Fig 8


G3PDH
LAT
D47 V47 V48 +
D47 V47 V48 +


IL-10
Ifn-gamma
D47 V47 V48 +
D47 V47 V48 +


RANTES
D47 V47 V48 +

BRAIN, BEHAVIOR, AND IMMUNITY 8, 185–203 (1994)

Chronic Morphine Treatment Suppresses CTL-Mediated Cytolysis, Granulation, and cAMP Responses to Alloantigen[1]

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Exposure to opioid drugs (e.g., morphine) *in vivo* has been shown to suppress natural killer cell activity. However, the effects of *in vivo* exposure to opioids on cytotoxic T lymphocyte (CTL) activity has not been investigated. The administration of morphine (50.0 mg/kg, sc) to alloimmunized mice for 11 days resulted in a significant decrease in peritoneal and splenic CTL activity. Moreover, the intracellular content of serine esterases and esterase release by $CD8^+$ effector cells from chronic morphine-treated mice was reduced compared to that of effector cells from vehicle-treated controls. In addition, the $CD8^+$ cAMP response to alloantigen was diminished compared to $CD8^+$-enriched cells from vehicle-treated animals. However, conjugate formation between effector and target and subsequent killing of target by effector cells did not reveal significant differences between vehicle- and chronic morphine-treated animals. Serum corticosterone and dehydroepiandrosterone levels were significantly lower in the chronic morphine-treated animals while proopiomelanocortin gene expression (exon 3) in splenic lymphocytes did not correlate with morphine-mediated suppression of CTL activity. These results indicate that CTL activity is sensitive to chronic morphine exposure, implicating opioids as important cofactors during viral infections in suppressing cell-mediated immunity.

INTRODUCTION

Short-term (<120 h) morphine administration has been shown to reduce natural killer (NK)[3] activity (Shavit, Lewis, Terman, Gale, & Liebeskind, 1984; Bayer, Daussin, Hernandez, & Irvin, 1990), impair immunoglobulin production (Bussiere, Adler, Rogers, & Eisenstein, 1992; Pruett, Han, & Fuchs, 1992), suppress phagocytic activity (Levier, Brown, McCay, Fuchs, Harris, & Munson, 1993; Szabo, Rojivin, Bussiere, Eisenstein, Alder, & Rogers, 1993), and induce thymic

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[3] Abbreviations used: ACTH, adrenocorticotropic hormone; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; CTL, cytotoxic T lymphocyte; DHEA, dehydroepiandrosterone; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunoabsorbent assay; EPI, epinephrine; FACS, Fluorescence activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HBSS, Hank's buffered saline solution; HSV-1, herpes simplex virus type-I; HPA, hypothalamic-pituitary adrenal; ICAM, intracellular adhesion molecule; IFN, interferon; IL, interleukin; ip, intraperitoneal: LU, lytic unit; NE, norepinephrine; NK, natural killer; PEL, peritoneal exudate leukocytes; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLC, phospholipase C; POMC, Proopiomelanocortin; RT, reverse transcription; sc, subcutaneous; SL, splenic lymphocyte; SNS, sympathetic nervous system; Triton X-100, *t*-octylphenoxypolyethoxyethanol.

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hypoplasia (Fuchs & Pruett, 1993). In monkeys and humans, chronic morphine use is known to suppress NK activity (Novick, Ochshorn, Ghali, Croxson, Mercer, Chiorazzi, & Kreek, 1989; Carr & France, 1993).

The action of opioids on the nervous system has been proposed as the route to immunomodulation. Studies have shown that morphine can act centrally (Shavit, Depaulis, Martin, Terman, Pechnick, Zane, Gale, & Liebeskind, 1986) through receptors located in the periaqueductal gray matter of the mesencephalon (Weber & Pert, 1989). Two central nervous system (CNS) pathways have been shown to be involved in opiate-induced immunomodulation, the hypothalamic pituitary, adrenal (HPA) axis, and the sympathetic nervous system (SNS). The HPA axis has been implicated in short-term studies of opioid-induced immunosuppression through the release of corticosterone (Bryant et al., 1991; Sei, Yoshimoto, McIntyre, Skolnick, & Arora, 1991; Pruett et al., 1992; Fuchs et al., 1993; Migliorati, Nicoletti, D'Adamio, Spreca, Pagliacci, & Riccardi, 1994). Endogenous corticosterone is selectively immunosuppressive suppressing interleukin (IL)-1, IL-2, and interferon (IFN) gamma production but acting synergistically with γ-IFN in enhancing monocyte activation and the generation of reactive oxygen intermediates (for review Munck & Guyre, 1991). Corticosterone has also been implicated as the primary mediator of opioid-induced apoptosis of thymocytes resulting in thymic hypoplasia, a process reversible by adrenalectomy and mimicked by dexamethasone (Sei et al., 1991). Furthermore, dexamethasone has been shown to induce apoptosis in mature NK and cytotoxic T lymphocyte (CTL) cells *in vitro* and the addition of IL-2 or IL-4 protects against apoptosis (Migliorati et al., 1994).

The SNS is implicated in some forms of morphine-mediated immunosuppression through adrenergic pathways. Specifically, β-adrenoceptor antagonists have been shown to block morphine-mediated suppression of mitogen-induced lymphocyte proliferation (Fecho, Dykstra, & Lysle, 1993). α-Adrenergic antagonists (and to a lesser extent, β-adrenoceptor antagonists) have been shown to block suppression of splenic NK activity following acute morphine administration (Carr, Gebhardt, & Paul, 1993). Furthermore, recent data suggest that central rather than peripheral adrenergic pathways are involved in morphine-mediated suppression of splenic NK activity (Carr, Mayo, Gebhardt, & Porter, 1994a). These observations coincide with previous data showing the intracisternal administration of morphine resulted in the elevation of serum norepinephrine (NE), epinephrine (EPI), and dopamine from SNS stimulation of the adrenal medulla. The increase in monoamines was blocked by naloxone and the selective deinnervation of the adrenals (Van Loon, Appel, & Ho, 1981; Appel, Kirtsy-Roy, & Van Loon, 1986). Autonomic innervation of primary and secondary lymphoid tissue (Felten, Felten, Carlson, Olschowka, & Livnat, 1985) and the presence of α- and β-adrenergic receptors on lymphocytes have been demonstrated (McPherson, & Summers, 1982; Fuchs, Albright, & Albright, 1988), suggesting direct effects of catecholamines on cells of the immune system. Moreover, a decrease in the affinity and increase in the number of β-adrenergic receptors has been reported following acute morphine administration (Baddley, Paul, & Carr, 1993) supporting the notion of an intricate relationship between the adrenergic system and opioid-induced immunomodulation. Some immunomodulatory effects of morphine have also been found to be specific to the splenic but not to mesenteric lymph node lymphocytes, suggesting a high degree of compartmentalization consistent with SNS innerva-

tion of lymphoid tissue (Baddley et al., 1993; Lysle, Coussons, Watts, Bennett, & Dykstra, 1993).

Finally, the presence of opioid binding sites on lymphoid cells suggests a direct pathway of immunomodulation. Receptors for opioids are known to exist on lymphocytes (Madden, Donahoe, Zwemer-Collins, Shafer, & Falek, 1987; Carr, DeCosta, Kim, Jacobson, Guarcello, Rice, & Blalock, 1989; Ovadia, Nitsan, & Abramsky, 1989). These receptors have functional importance in immune homeostasis as indicated by *in vitro* measurement of immunocompetence (for review Carr, 1991).

The focus of this study was to assess the effect of chronic morphine exposure *in vivo* on the effector mechanisms of cell-mediated immunity, NK and CTLs measured *in vitro*. An 11-day chronic morphine treatment protocol was developed to investigate this question as well as address the significance of neuroendocrine influences on cell-mediated immunity. The intent of this initial study is to address long-term pain management, addiction, and withdrawal on immune homeostasis.

MATERIALS AND METHODS

Mice and Tumor Lines

Female C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) and C3H/HeN (Harlan-Sprague Dawley, Indianapolis, IN) mice were housed in groups of 6–10 per cage and maintained on a 12-h light/dark cycle. Access to water and food (Purina Mouse Chow) was available *ad libitum*. The YAC-1 mouse lymphoma cell line, P815 mastocytoma cell line, and EL-4 lymphoma cell line were obtained from the American Type Culture Collection (Rockville, MD); the cells have been maintained in culture by biweekly passage over the course of no more than 6 months/frozen lot.

Morphine Treatment Regimen

A dose–response study has established that 50.0 mg/kg of morphine sc results in maximal suppression of NK cytolytic activity (Carr, Gerak, & France, 1994b). In addition, preliminary results from dose effect studies indicates that 50.0 mg/kg elicits maximum suppression of CTL activity in alloimmunized mice (Carr, unpublished observation). This dose was used in all experiments.

In the chronic morphine exposure protocol, C3H/HeN mice (n = 23/group) were administered vehicle or morphine 2 h prior to receiving 1×10^7 C57BL/6J spleen cells, intraperitoneally (ip). Following the immunization, mice received vehicle or morphine daily for an additional 6 days. On Day 7, mice were reimmunized with 1×10^7 C57BL/6J spleen cells ip 2 h after the administration of vehicle or morphine. Following the second immunization, mice received morphine or vehicle daily for an additional 3 days. On Day 11, the mice were sacrificed and splenic lymphocytes (SL) were recovered and assayed for CTL and NK activity or processed further for other assays described below.

Lymphocyte Preparation

All mice were sacrificed by CO_2 asphyxiation and peritoneal lavage was performed using 10 ml of sterile Hank's buffer saline solution (HBSS). Cells were collected by recovering 10 ml of peritoneal fluid through a 20-gauge needle and 10-ml syringe. Spleens were removed and cell suspensions were prepared by

mechanical dispersion. SL and peritoneal exudate leukocytes (PEL) were washed with HBSS (250*g*, 5 min). Red blood cells were osmotically lysed using 0.84% NH_4CL; the cells were subsequently washed with HBSS (250*g*, 5 min) and resuspended in RPMI-1640 containing 10% fetal calf serum (FCS) and 2.5% Hybrimax (Sigma Chemical Co., St. Louis, MO) antibiotic (100 units/ml penicillin; 200 μg/ml streptomycin)/antimycotic (250 ng/ml amphotericin B) solution (complete medium). Cells were counted and examined for viability via trypan blue exclusion dye.

^{51}Cr-Release Cytolytic Assay

CTL activity was assayed using a 4-h microcytotoxicity assay with ^{51}Cr-labeled EL-4 (H-2^b) cells as targets. Between 5×10^4 and 160×10^4 effector cells were mixed with 1×10^4 target cells in conical 96-well microtiter plates (Costar, Cambridge, MA) in a reaction volume of 0.2 ml of complete medium. The cultures were incubated 4 h at 37°C in a 5% CO_2 atmosphere. A 100-μl aliquot of cell-free supernate was taken from each well and its ^{51}Cr content was determined using a Beckman gamma counter. The cytolytic activity was determined as follows: percentage cytolytic activity = [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(total cell-associated ^{51}Cr release − spontaneous ^{51}Cr release)] × 100 where "spontaneous" refers to ^{51}Cr release by target cells in the absence of effector cells. Total cell-associated ^{51}Cr was determined by measuring the ^{51}Cr content in the supernates of 10^4 target cells incubated at 37°C in a 5% CO_2 atmosphere in the presence of 0.1% *t*-octylphenoxypolyethoxyethanol (Triton X-100, Sigma Chemical Co.) in complete medium or measuring the ^{51}Cr content in 10^4 ^{51}Cr-labeled target cells. Spontaneous release was consistently between 10 and 15%. Each effector to target cell ratio was measured in triplicate/animal. One lytic unit (LU) was defined as the number of effector cells required to lyse 20% of the target cells per 10^7 total SL or PEL population. To determine antigen specificity for the CTL assay, P815 (H-2^d) mastocytoma cells were ^{51}Cr-labeled and used as third-party targets in the 4-h microcytotoxicity assay.

CTL Serine Esterase Assay

One hundred microliters of SL at a concentration of 2×10^6 cells/ml from C3H/HeN mice were placed in duplicate into 96-well conical-bottom microtiter plates. 10^5 irradiated (900 rads) C57BL/6J spleen cells in 100 μl of complete media were added to one set of wells. One hundred microliters 0.2% Triton X-100 in complete media was added to the other set as a measure of total activity. Cells were incubated for 2 or 4 h and 10 μl of supernate was removed from each well and transferred to the wells of a separate 96-well flat-bottom plate (Costar). To each well 190 μl of substrate consisting of 0.2 m*M* *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl ester (Sigma Chemical Co., St. Louis, MO) was added. Duplicate wells of complete media and 0.2% Triton X-100 were included as reagent blanks. The plates were incubated for 1 h and the optical density at 405 nm determined in a Dynatech MR5000 automatic plate reader. The esterase activity was determined as follows: percentage esterase activity = [(experimental absorbance − complete media blank absorbance)/(total absorbance-0.2% Triton X-100 absorbance)] × 100. Each well was read in triplicate.

$CD8^+$ Lymphocyte Enrichment

Mouse T cell subset enrichment columns (R & D Systems, Minneapolis, MN) were prepared as suggested by the manufacturer. SL from the groups were pooled as were SL from the morphine treatment groups and separately applied to CD8 enrichment columns. Recovered T cells were then assayed for CTL activity using ^{51}Cr-labeled EL-4 cells as targets in the ^{51}Cr-release cytolytic assay. Unfractionated splenic lymphocytes consisted of 12 ± 3% $CD8^+$ lymphocytes, whereas the $CD8^+$-enriched SL population consisted of 64 ± 5% $CD8^+$ lymphocytes, 0.0% $CD4^+$ lymphocytes, and 32 ± 6% B lymphocytes, macrophages, and null cells as determined by flow cytometry using a Coulter Elite (data not shown).

Serum Corticosterone and Dehydroepiandrosterone (DHEA) Assay

Animals were sacrificed by CO_2 asphyxiation and the thoracic cavity immediately opened and blood extracted via heart puncture with a 1-ml syringe and 27½-gauge needle. Samples were placed on ice overnight and exposed to the air to favor clot formation. Tubes were then spun at 10,000*g* for 1 min in a microcentrifuge (IEC; Needham, MA). Sera were removed and frozen at −20°C for later assay.

Sera from sacrificed animals were assayed for corticosterone by RIA using a corticosterone kit specific for rat and mouse corticosterone (ICN Biomedicals; Costa Mesa, CA) or DHEA (Diagnostic Products Corp., Los Angeles, CA). All samples were assayed simultaneously in duplicate. Standards were run simultaneously with experimental samples. The concentration of serum corticosterone and DHEA in the experimental samples was extrapolated from the curve generated from the known standards. The standard curve typically had a corresponding coefficient for linearity >.9900.

Cellular Cyclic Adenosine Monophosphate (cAMP) Assay

Enriched $CD8^+$ lymphocytes (10^6) in 200 μl of complete media were placed into two Falcon 2054 (Becton Dickinson; Lincoln Park, NJ) sterile snap cap tubes. To one of these tubes, 200 μl of complete medium was added (effector only, unstimulated); to the other tube was added 10^4 irradiated (900 rads) EL-4 target cells (effector + target, stimulated) in 200 μl of complete medium. A further tube containing 10^4 irradiated target cells in 400 μl of complete medium (target associated cAMP) was also prepared. All tubes were incubated at 37°C/5% CO_2 for 30 min. Parallel experiments were carried out representing vehicle and chronic morphine treatment groups. Following incubation, cells were immediately assayed for cAMP by enzyme-linked immunoabsorbent assay (ELISA) (ELISA Technologies; Lexington, KY). Standards were run simultaneously with experimental samples. The concentration of cAMP in the experimental samples was extrapolated from the curve generated from the known standards. The standard curve typically had a correlation coefficient for linearity >.9900.

Conjugate Formation Fluorescence-Activated Cell Sorter (FACS) Analysis

The protocol for labeling and measuring conjugates was carried out as described (Lebow & Bonavida, 1990) with modifications. Specifically, 5×10^5 $CD8^+$ lymphocytes from each treatment group (vehicle and chronic morphine) were placed into Falcon 2054 (Becton Dickinson; Lincoln Park, NJ) sterile snap cap tubes. An

additional tube of 5×10^5 enriched cells from vehicle-treated animals was also prepared (effector only). Cells were washed once with 1 ml PBS at 250*g* for 5 min, room temperature. Fluorescein isothiocyanate (FITC) dye was prepared by dissolving powdered FITC (Sigma, St. Louis, MO) in dimethyl sulfoxide (DMSO) at a concentration of 50 m*M* and diluting 1:1000 in phosphate-buffered saline (PBS, pH 7.4). Dye solution (100 μl) was added to the decanted washed cells and the cells then vortexed momentarily before being incubated at 37°C/5% CO_2 for 10 min with the caps dislodged. Cells were then washed twice as before with PBS and decanted. A volume of 250 μl of target cells (EL-4) at a concentration of 4×10^6 cells per milliliter of complete media was added to the cells from the experimental groups establishing an effector to target ratio of 1:2, favoring formation of single effector–target conjugates. Complete medium (250 μl) alone was placed into the tube containing effector cells alone and an additional tube containing 250 μl of the target cell suspension was prepared (target alone). Tubes were incubated for 90 min at 37°C/5% CO_2 with the caps dislodged and occasionally agitated gently to maintain suspension. Following the incubation, the cells were immediately placed on ice for FACS analysis. Immediately prior to FACS analysis, 5 μl of 0.1 μg/μl solution in phosphate-buffered saline (PBS, pH 7.4) of propidium iodide (Sigma) was added and the sample gently agitated to distribute the dye and ensure a uniform cell suspension.

FACS analysis was carried out on a Coulter Elite FACS (Coulter, Hialeah, FL). A log forward scatter vs log side scatter plot was used to gate viable cells for analysis, thereby separating whole cells from cell fragments. Whole effector only cells were plotted log green (FITC) vs log yellow (propidium iodide) which allowed effectors to be sorted for viability, needed for calculation of percentage conjugation in subsequent analysis of experimental samples. A log green vs log side scatter plot of effector only was gated so that viable effector cells were counted. The target-only sample was then analyzed with the above gates to ensure that target alone was not counted in the effector population. The vehicle experimental sample was analyzed and another gate was created to encompass the bulk of conjugates. An analysis window, cell count vs log yellow, was gated to the conjugates to allow for the calculation of percentage killing within the conjugated population. Analysis of the chronic morphine-treated sample proceeded with the above gate settings.

Calculation of percentage conjugation: percentage conjugation = (No. of conjugates/[No. of viable effectors + No. of conjugates]) × 100. Calculation of percent killing: percentage killing = (No. of gated dead cells within conjugate population/total gated conjugates) × 100. Effector cells only yielded 5 ± 2% uptake of PI. This number was used to subtract out percentage killing in the conjugate gate. Target cells only showed less than 1% PI uptake and were therefore not included in the background subtraction.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

RNA extraction. SL (1×10^7) RNA was extracted by vortexing in 1 ml of UltraSpec (Biotex, Houston, TX) RNA extraction solution in 1.5-ml microcentrifuge tubes. Samples were placed on ice for 5 min after which 0.2 ml chloroform was added followed by 15 s of vortexing and another 5-min incubation on ice. Samples were vortexed and then centrifuged for 15 min at 12,000*g* and 4°C. After centrifugation, 80% of the upper aqueous phase was removed and placed into

another 1.5-ml microcentrifuge tube. One volume of ice-cold isopropanol was added to the aqueous phase and the mixture was vortexed for 10 s and placed on ice for 20 min. The samples were then centrifuged for 10 min at 12,000*g* and 4°C. Samples were vacuum decanted and washed twice with ice-cold 75% ethanol, centrifuging for 10 min at 7500*g* and 4°C after each wash. Samples were then dried under vacuum and resuspended in 75 μl of sterile water. Optical density readings (Beckman DU-50 spectrophotometer) at 260 and 280 nm of a 1-μl aliquot were taken to determine the concentration and purity of RNA.

Reverse transcription. RNA samples were diluted to a concentration of 1 μg/3 μl water, 3 μl of each were placed into 0.5 ml microcentrifuge tubes suitable for temperature cycling. A RT master mix was prepared with the following reagents (Perkin Elmer Cetus, Norwalk, CT.) at these corresponding final concentrations: $MgCl_2$, 5 m*M;* 10× PCR buffer, 1×; dGTP, 1 m*M;* dCTP, 1 m*M;* dATP, 1 m*M;* dTTP, 1 m*M;* RNase inhibitor, 1 U/μl; RT, 2.5 U/μl; random hexamer primers, 2.5 μ*M*. A 17-μl aliquot of the master mix was added to each of the 1-μg samples. Tubes were submitted to temperature cycling (MJ Research) consisting of 42°C for 15 min followed by 99°C for 5 min and 5°C for 5 min.

Polymerase chain reaction. A PCR master mix was prepared with the following reagents (Perkin Elmer Cetus) at these corresponding final concentrations: $MgCl_2$, 2 m*M;* 10× PCR buffer, 1×; *Taq* DNA polymerase, 2.5 U/μl; relevant upstream primer 0.15 μ*M;* relevant downstream primer, 0.15 μ*M*. An 80-μl aliquot of the master mix was added to each of the samples which had been reverse transcribed. Tubes were again submitted to temperature cycles of 95°C for 2 min followed by 35 cycles of 1 min at 95°C and 1 min at 65°C followed by 7 min at 60°C and storage at 4°C.

Primers

G3PDH

5′–GTC–ATG–AGC–CCT–TCC–ACG–ATG–C–3′ upstream

5′–GAA–TCT–ACT–GGC–GTC–TTC–ACC–3′ downstream

POMC

5′–GAG–ATG–AAC–AGC–CCC–TGA–CTG–AAA–AC–3′ upstream

5′–AAT–GAG–AAG–ACC–CCT–GCA–CCC–TCA–CTG–3′ downstream

Route and Dose of Morphine and Herpes Simplex Virus-I

Vehicle (sc) or morphine (50 mg/kg, sc) was administered to C3H/HeN mice. Two hours following the drug administration, the LD_{50} of the McKrae strain of herpes simplex virus (HSV)-I (3×10^5 PFU) in RPMI-1640 was administered in the footpad of the mice in a volume of 50 μl. Subsequent to the virus administration, mice received vehicle (sc) or morphine (50 mg/kg, sc) daily up to the time of death of the animal or until the end of the observation period (21 days).

Reagents

Morphine sulfate was provided by the Research Technology Branch of The National Institute on Drug Abuse (Rockville, MD). Drug was dissolved in DMSO and diluted with HBSS to a concentration containing 25% DMSO. A volume of 100 μl of this solution containing the drug at the appropriate concentration was delivered to each mouse. Vehicle consisted of 25% DMSO in HBSS.

Statistics

One-way ANOVA (Randomized Block Design) was used together with Scheffé or Tukey's post hoc multiple comparisons test to determine significance ($p < .05$) between vehicle- and drug-treated groups. In some experiments, Bonferonni's t test was used to determine significance ($p < 0.05$) between vehicle- and drug-treated groups. In addition, the nonparametric Wilcoxon-signed rank test was used to calculate significant differences between the treated groups of animals. This statistical package used the GBSTAT program (Dynamic Microsystems Inc., Silver Springs, MD).

RESULTS

Chronic Morphine Exposure Suppresses CTL Activity

Mice treated with morphine for 11 days exhibited significantly less SL CTL activity compared to vehicle-treated controls (Fig. 1). PEL CTL activity was also significantly lower in the chronic morphine-treated compared to vehicle-treated mice (Fig. 2). However, both vehicle- and chronic morphine-treated mice showed similar levels of splenic NK activity (Fig. 3). SL from chronic morphine- and vehicle-treated mice were also assayed for lysis of a third-party target. Neither population of PEL or SL showed any measurable cytolytic activity against ^{51}Cr-labeled P815 cells (data not shown). In addition, SL from unprimed (nonimmunized) mice had no measurable CTL activity to the ^{51}Cr-labeled EL-4 targets (data not shown). Consistent with another study (Carpenter & Carr, submitted for publication) splenic CD8$^+$-enriched effector cells taken from mice treated daily with morphine over 11 days showed significantly less CTL activity compared to vehicle-treated controls (Fig. 4).

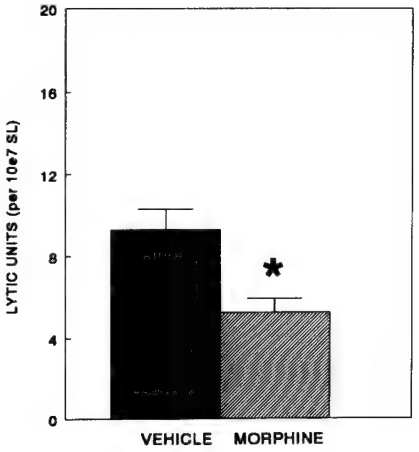

FIG. 1. Chronic morphine exposure suppresses splenic lymphocyte CTL activity. C3H/HeN mice (n = 23/group) were administered morphine (50.0 mg/kg, sc) or vehicle 2 h prior to alloimmunization (1×10^7 C57BL/6 SL, ip). Mice received morphine (50.0 mg/kg, sc) or vehicle daily for 9 days and were reimmunized 7 days after the primary immunization. Mice were sacrificed on Day 11 and the SL were assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. *$F(1,22) = 15.455$, $p < .05$ comparing vehicle- to chronic morphine-treated mice as determined by one-way ANOVA and Scheffe multiple comparison test. Bars represent SEM, $n = 23$.

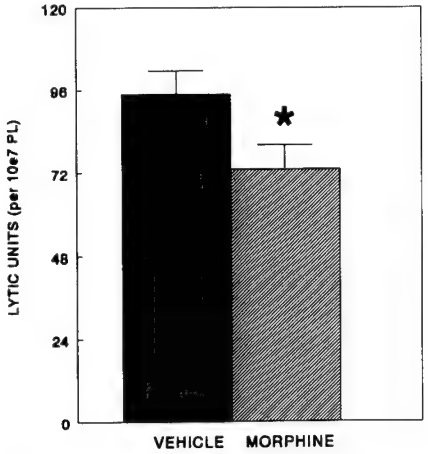

FIG. 2. Chronic morphine exposure suppresses CTL activity of peritoneal exudate leukocytes. Mice were treated as described in the legend of Fig. 1. PEL were collected and assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. *$F(1,22) = 6.3882$, $p < .05$ comparing vehicle- to chronic morphine-treated mice as determined by ANOVA followed by Scheffe multiple comparison test. Bars represent SEM, $n = 23$.

Serine Esterase Release Is Reduced in Response to Antigen in SL from Chronic Morphine-Treated Animals

Serine esterases are contained within the granules of CTLs and are released upon contact with target (Pasternack & Eisen, 1985; Pasternack, Verret, Liu, & Eisen, 1986; Young, Leong, Liu, Damiano, & Cohn, 1986). To further investigate the reduced cytolytic activity found in the chronic morphine-treated animals, serine esterase release and granulation was measured. Serine esterase release from SL in response to alloantigen stimulation yielded results which paralleled the SL CTL activity. Specifically, after 2 h exposure to irradiated targets (C57Bl/6J splenocytes) the percentage of serine esterase content in the supernates was significantly lower in the SL taken from chronic morphine-treated mice com-

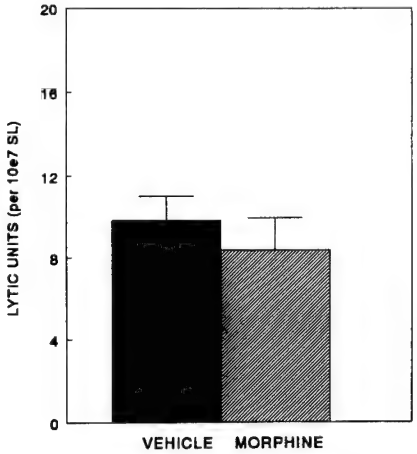

FIG. 3. Chronic morphine exposure has no effect on splenic NK activity. Mice were treated as described in the legend of Fig. 1. SL were assayed for NK activity using ^{51}Cr-labeled YAC-1 cells. Bars represent SEM, $n = 23$.

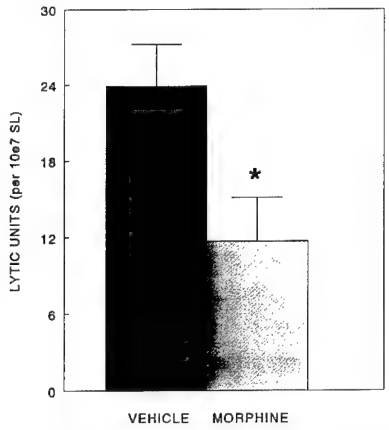

FIG. 4. Chronic morphine exposure suppresses $CD8^+$-enriched CTL activity. C3H/HeN mice (n = 4/group) were treated as described in the legend of Fig. 1. Mice were sacrificed on Day 11 and the SL were pooled within treatment groups, enriched for $CD8^+$ cells, and assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. *$F(1,5) = 6.5794$, $p < .05$ comparing vehicle- to chronic morphine-treated mice as determined by ANOVA and Scheffe multiple comparison test. Bars represent SEM, $n = 4$.

pared to serine esterase release from the vehicle-treated controls (Table 1). In addition, there was a significant decrease in the total serine esterase content of SL taken from chronic morphine compared to vehicle-treated mice at the 2-h time point (Table 1). By 4 h postincubation, the differences were no longer significant (Table 1).

Cyclic AMP Levels of Enriched $CD8^+$ Cells from Chronic Morphine-Treated Animals Revealed a Decreased Response to Antigen

Cyclic AMP has been implicated as the second messenger responsible for the termination of attack between effector and target (Valitutti, Dessing, & Lanza-

TABLE 1
Morphine Suppresses the Release and Total Cell-Associated Serine Esterase (SE) Level in CTL Cells[a]

	SE content in supernatant[b]		Cell-associated SE[c]	
Treatment	2-h	4-h	2-h	4-h
Vehicle	13.3 ± 2.2	25.5 ± 4.7	.097 ± .008	.046 ± .007
Morphine	8.4 ± 1.9*	18.4 ± 3.7	.066 ± .002*	.039 ± .004

[a] Splenic lymphocytes from immunized mice were assayed for SE content following reexposure to antigen.

[b] Cell-free supernatant from mixed lymphocyte reactions were collected and assayed for SE at the designated time points. The numbers represent the percentage of the total cell-associated SE. Splenic lymphocytes from unprimed C3H/HeN mice were used to determine baseline SE levels. The baseline levels were subtracted from the experimental percentages.

[c] Total cell-associated SE was determined following lysis (0.1% Triton X-100 in complete medium) of the C3H/HeN splenic lymphocytes. Numbers are in absorbance read at 405 nm (background subtracted).

* $F(1,11) = 6.3307$, $p < .05$ for the percentage SE content in supernatant; $F(1,4) = 9.9415$, $p < .05$ for cell-associated SE comparing morphine-treated to vehicle-treated controls as determined by one-way ANOVA and Scheffe multiple comparison test.

recchia, 1993). A rise in intracellular cAMP has been associated with detachment from the target cell and a halt in degranulation ("preservation of granulation") (Valitutti et al., 1993). Cyclic AMP has also been implicated as the signal responsible for the initiation of recycling of CTL for subsequent lytic function (Valitutti et al., 1993).

To further examine mechanisms involved in the suppression seen in the chronic morphine-treated animals, measurements of intracellular cAMP in enriched $CD8^+$ cells were made in response to antigen stimulation *in vitro*. Results indicate that the increase in cAMP of $CD8^+$ cells derived from chronic morphine-treated animals is reduced in response to target cells when compared to the response in the $CD8^+$-enriched effector cells from vehicle-treated animals (Table 2).

Ability of $CD8^+$ Cells Derived from Chronic Morphine-Treated Mice to Form Conjugates Is Not Significantly Impaired

In an attempt to correlate conjugate formation with reduced cytolytic activity, a study of the ability of the cells to form conjugates and kill targets was undertaken. The results revealed no significant differences in conjugate formation in the cells derived from chronic morphine-treated versus vehicle-treated animals following a 90-min incubation period. Of the gated events (viable effector cells conjugated to targets), 45.1 ± 1.8% were conjugated to target cells of the enriched $CD8^+$ effector cells from vehicle-treated mice compared to 40.7 ± 5.5% of the effector cells from the chronic morphine-treated group. Of the cells engaged in conjugation, 3.4 ± 1.1% of the conjugated targets were dead in the vehicle group compared to 3.1 ± 0.7% in the morphine group. Consistent with these results, cell surface expression of the CD11a adhesion molecule necessary for the formation of effector–target conjugates was similar in both $CD8^+$ SL from vehicle- and chronic morphine-treated animals (Table 3).

TABLE 2
Chronic Morphine Exposure Attenuates cAMP Production Following Antigen Stimulation in $CD8^+$ Effector Cells

Experiment	Treatment	Effector only	Effector + target
1	Vehicle	8.9 ± 1.8[a]	13.2 ± 0.8
	Morphine	10.3 ± 1.7	10.7 ± 2.1
2	Vehicle	9.1 ± 0.1	11.0 ± 0.7
	Morphine	5.0 ± 1.9	5.0 ± 2.0
3	Vehicle	10.1 ± 0.7	16.9 ± 2.8
	Morphine	9.0 ± 0.7	14.1 ± 3.7
4	Vehicle	6.2 ± 0.9	11.6 ± 0.5
	Morphine	5.6 ± 0.8	6.5 ± 0.6
Summary	Vehicle	8.6 ± 0.8	13.2 ± 1.3*
	Morphine	7.5 ± 1.3	9.1 ± 2.1

[a] Numbers are expressed in pmols/10^6 effector cells ± SEM, $n = 4$. Target cells alone yielded 1.1 ± 0.5 pmol/10^4 cells.

* $F(3,15) = 2.9738$, $p < .05$ comparing effector only to effector + target in the summary vehicle group as determined by one-way ANOVA and Tukey's post hoc t test.

TABLE 3
Expression of CD11a on $CD8^+$ Lymphocytes Is Not Altered after Chronic Morphine Exposure

Treatment	Percentage $CD11a^{+a}$	Percentage $CD11^+CD8^+$
Vehicle	75.9 ± 0.6	10.5 ± 0.6
Morphine	81.8 ± 0.9*	12.5 ± 0.6

[a] Total percentage of $CD11a^+$ expressing splenic lymphocytes ± SEM, $n = 6$ vehicle-treated, $n = 5$ morphine-treated.

* $p < .05$ comparing morphine-treated to vehicle-treated mice as determined by Bonferonni's t test.

Serum Corticosterone Levels Are Significantly Lower after Chronic Treatment with Morphine

To investigate the role of corticosterone in the suppression of cytolytic activity resulting from chronic morphine exposure, sera were assayed for corticosterone in parallel with cytolytic assays of $CD8^+$-enriched cells. Serum corticosterone levels in vehicle-treated animals were significantly elevated compared to those of morphine-treated mice (Fig. 5A). Likewise, DHEA levels were decreased in the chronic morphine-treated mice compared to the vehicle-treated controls (Fig. 5B).

Expression of POMC in SL Does Not Correlate with Chronic Morphine- or Vehicle-Treated Animals

Two of the important products of the POMC gene are adrenocorticotropic hormone (ACTH) and opioid peptides (e.g., α, β, and γ endorphins). An alternative source of ACTH may be lymphocytes (Smith, Meyer, & Blalock, 1982). To investigate these cells as a source of immunomodulation, the expression of the POMC gene in the SL was undertaken. SL RNA from individual animals within the chronic morphine and vehicle experimental groups was submitted to RT.

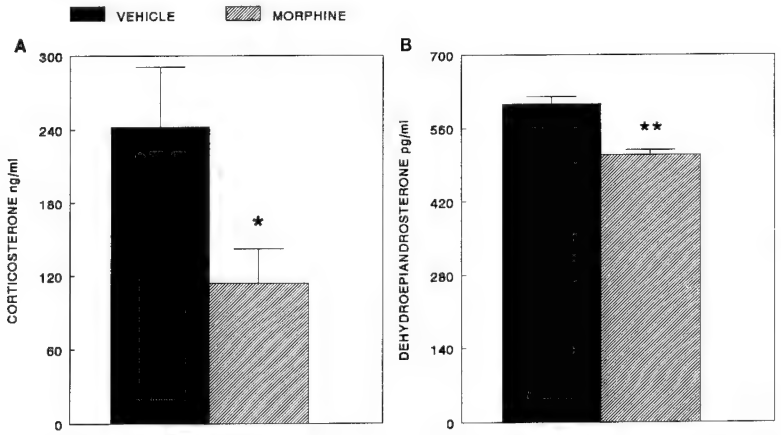

FIG. 5. Chronic morphine exposure reduces serum corticosterone and serum DHEA levels. C3H/HeN mice were treated as described in the legend of Fig. 1. Upon sacrifice, blood was obtained by cardiac puncture and assayed for corticosterone and DHEA level by radioimmunoassay. (A) Analysis of serum corticosterone levels. *$p < .05$ comparing vehicle- to chronic morphine-treated mice as determined by Bonferroni's t test. Bars represent SEM, $n = 11$ (vehicle) or 13 (chronic morphine) animals tested. (B) Analysis of serum DHEA levels. **$F(1,29) = 32.0516$, $p < .01$ comparing vehicle- to chronic morphine-treated mice as determined by ANOVA and Scheffe multiple comparison test. Bars represent SEM, $n = 15$/group.

Products of reverse transcription were then amplified by PCR using primers specific for G3PDH and Exon 3 of POMC. Positive control RNA (from AtT-20 corticotroph cell line) was reverse transcribed and amplified in parallel with experimental samples. No pattern could be discerned comparing the drug- to vehicle-treated groups (Fig. 6). Specifically, lymphocytes from 3 of 13 vehicle animals were positive for exon 3 POMC-amplified product, while the lymphocytes of 6 of 14 chronic morphine-treated animals screened were positive. All animals had equivalent levels of G3PDH amplified product (Fig. 6).

Chronic Morphine Exposure Reduces the Survival Rate of C3H/HeN Mice Infected with Herpes Simplex Virus Type I(HSV-1)

To further define the biological significance of chronic morphine exposure, C3H/HeN mice were infected with an LD_{50} of the McKrae strain of HSV-1. One of 12 chronic morphine-treated mice infected with the virus survived the 21-day observation period (Fig. 7). Moreover, 9 of 12 morphine-exposed mice had succumbed to the infection within 7 days of virus administration. In comparison, 3 of 12 vehicle-treated mice survived the virus infection with 6 of 12 mice succumbing to infection 7 days following virus administration.

DISCUSSION

In the present study, we have investigated the immunomodulatory effect of chronic morphine exposure on NK and CTL activity. Short-term (daily exposure to morphine for 5 days) morphine (50.0 mg/kg, sc) administration did not modify CTL activity in alloimmunized C3H/HeN mice (Carr, unpublished observation). In addition, the generation of CTLs in *in vitro* one-way mixed lymphocyte reactions is unaffected in the presence of morphine (10^{-5}–10^{-11} *M*) (Carr & Carpenter, submitted), suggesting that morphine does not directly act on immune cells in the context of CTL generation. However, chronic morphine exposure does sup-

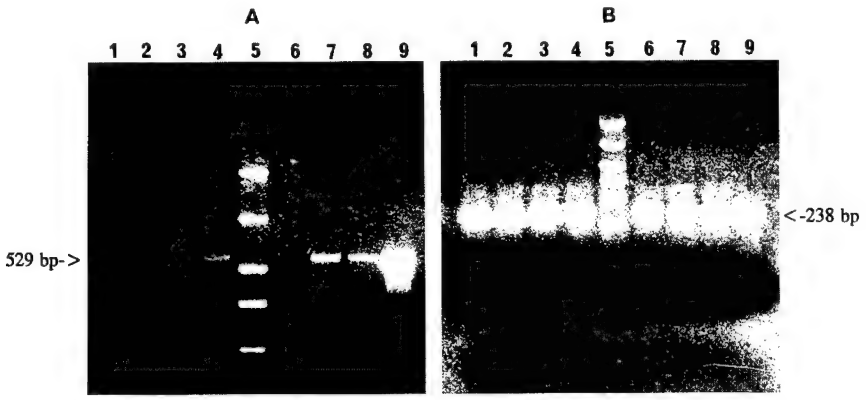

FIG. 6. POMC transcript levels do not coincide with morphine-mediated suppression of splenic CTL activity. SL RNA was subjected to RT-PCR using oligonucleotide probes specific for exon 3 of POMC or G3PDH as described under Materials and Methods section. Lanes 1–4, RNA from SL from vehicle-treated mice; lane 5, DNA ladder in descending order: 1000, 700, 500, 400, 300, and 200 bp; lanes 6–8, RNA from SL from chronic morphine-treated mice; lane 9, RNA from AtT-20 pituitary tumor cells. (A) RT-PCR amplification using POMC primers resulting in a 529-bp product. (B) RT-PCR amplification using G3PDH primers resulting in a 238-bp product.

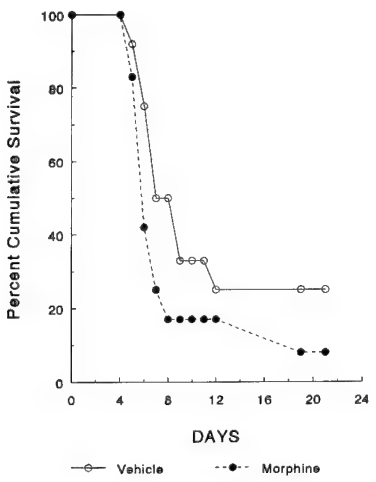

FIG. 7. The effect of chronic morphine treatment on the percentage of cumulative survival of C3H/HeN mice following HSV-I infection. Mice were injected into the footpad with the LD_{50} of the McKrae strain of HSV-I 2 h after receiving vehicle or morphine as described (see Materials and Methods). $p < .001$ comparing morphine-treated to vehicle-treated mice percentage survival as determined by the nonparametric Wilcoxon signed rank test, $z = 8.29$.

press CTL activity in alloimmunized mice and this effect is blocked by β-funaltrexamine (μ-selective opioid receptor antagonist) (Carpenter & Carr, submitted for publication) but not (*E*)-7-benzylidine-7-dihydronaltrexone (δ-selective opioid receptor antagonist) pretreatment (Carr & Carpenter, submitted for publication). Moreover, the suppression in CTL activity does appear to be modestly significant since only 8% of HSV-I-infected mice chronically treated with morphine survived the infection, while 25% of vehicle-treated HSV-I-infected mice survived. The reduction in percentage survival of vehicle-treated mice following the LD_{50} for this particular strain of HSV-I may be due to the daily handling and injections, resulting in a short-term "stressed state."

Compartmentalization of morphine-induced effects on immune functions is cited in some studies as evidence for the involvement of the SNS (Baddley et al., 1993; Lysle et al., 1993). This appears to be justified given the direct innervation of lymphoid tissue by fibers of the SNS (Felten, Felten, Bellinger, Carlson, Ackerman, Madden, Olschowka, & Livnat, 1987). The SNS uses norepinephrine almost exclusively as a mediator. The SNS, however, is not the sole source of norepinephrine. *In vivo,* the adrenal medulla produces epinephrine, dopamine, and norepinephrine and is controlled via the SNS as well. In addition, opioids are capable of activating this pathway (Van Loon et al., 1981; Appel et al., 1986). Accordingly, the global immunomodulatory effects of morphine could be mediated by SNS stimulation of the adrenal medulla, resulting in the release of sufficient quantities of catecholamines to exert a systemic immunomodulatory effect.

In vitro catecholamines have been shown to have immunomodulatory effects on indicators of CTL function. The lytic activity of CTL was found to be potentiated by the addition of NE, EPI, or isoproterenol (β-agonist) at the beginning of culture, coaddition of the β-blocker timolol abolished the augmentation (Felten et al., 1987; Livnat, Madden, Felten, & Felten, 1987). *In vivo,* chemical sympathectomy has been shown to reduce CTL activity and influence lymphocyte trafficking

(Madden & Livnat, 1991). Combined, the action of catecholamines suggest that their effect is dependent on concentration, timing, cell type, and site of action. For example, early effects on CTL activation and differentiation appear to potentiate activity, whereas late effects inhibit CTL effector function (Strom & Carpenter, 1980).

The effect of endogenous opioids on CTL generation *in vivo* has not been revealed. Unlike morphine, endogenous opioid peptides have been found to potentiate the generation of CTLs *in vitro* through a naloxone-sensitive mechanism (Carr & Klimpel, 1986). To investigate a possible role for these peptides (i.e., endorphines) *in vivo,* we examined the expression of the POMC gene by SL harvested from chronic morphine- and vehicle-treated animals. Results of RT-PCR amplifications of SL RNA failed to reveal a correlation between the expression of exon 3 POMC transcripts and the treatment group. This suggests that the POMC gene may be expressed only transiently in SL and is not an important component in the suppression of cytolytic activity found in the effector population.

The result showing a reduced corticosterone level in the chronic morphine-treated animals relative to vehicle-treated mice is unexpected. Previous studies have shown HPA axis involvement in modifying the immune system following short-term exposure to morphine (Bryant et al., 1991; Sei et al., 1991; Fuchs & Pruett, 1993). However, the present results would indicate the levels of corticosterone do not coincide with suppression of CTL activity. Moreover, recent results have shown short-term exposure to morphine (50 mg/kg daily for 5 days) *in vivo* has no effect on the generation of CTLs in alloimmunized C3H/HeN mice (Carr, unpublished observation), suggesting the influence of corticosterone in CTL generation or activity is minimal in this strain of mouse. However, in CBAxC57BL/6 mice, short-term exposure to morphine (50 mg/kg daily for 5 days) *in vivo* modifies CTL activity (Garza, Prakash, & Carr, submitted for publication), indicating the strain-specific nature of morphine-mediated immunomodulation as previously reported (Bussiere et al., 1992) as well as the potential role of the HPA axis.

In the present study, circulating levels of the adrenal cortical steroid hormone DHEA were found to be modestly but significantly lower in the morphine-treated mice. DHEA has previously been shown to protect mice against a lethal dose of virus (coxsackievirus and herpes simplex virus type II) (Loria, Inge, Cook, Szakal, & Regelson, 1988) and is predicted to interfere with the immunosuppressive effects of corticosterone (Riley, 1983). Recently, androstenediol, a metabolic product of DHEA has been found to be 100× more potent than DHEA in regulating resistance to viral and bacterial infections (Loria & Padgett, 1992). In terms of the present study, the results suggest that the increase in HSV-I-elicited encephalitis and death in chronic morphine-treated mice may be due in part to a reduction in the circulating levels of DHEA and metabolites, thus eliminating the endogenous corticosterone antagonist. However, the observation showing that corticosterone levels were also significantly lower in the chronic morphine-treated mice seems to suggest that the adrenal glands may not have been functioning correctly.

Early events in CTL target cell recognition include target cell adhesion followed by "programming" for lysis. Within 2–10 min following target cell adhesion cytoplasmic granules within the CTL reorients to the region near the interface with the target cell (Englehard, Gnarra, Sullivan, Mandell, & Gray, 1988). This pro-

gramming for lysis results from the activation of pathways involving phospholipase C (PLC), phosphatydylinositol turnover, and protein kinase C. A sharp increase in intracellular calcium from intracellular and extracellular sources promotes reorientation, fusion, and exocytosis of granules (Englehard et al., 1988; Ostergaard & Clark, 1987). Granule fusion is terminated simultaneously with a sharp increase in intracellular cAMP (Valitutti et al., 1993).

In an effort to identify the mechanism(s) for suppressed cytolytic activity, we investigated the ability of purified CTLs from chronic morphine- and vehicle-treated mice to form conjugates and subsequently lyse targets. A previous study showed that acute morphine administration suppressed conjugate formation and cytolysis of target cells by NK-enriched effector cells (Carr et al., 1994a). The results in the present investigation indicate that the ability of CTLs from chronic morphine-treated animals to form conjugates with allogeneic target cells at the 90-min time point is not impaired. This is consistent with the finding that there were no significant differences in CD11a expression by purified effectors cells between the vehicle- and chronic morphine-treated groups. Differences in CD11a expression would presumably result in differences in the avidity between effector–target conjugates resulting from the specific interactions between CD11a on effector cells and CD54 (ICAM-1) on the target cells (Dustin & Springer, 1989; Nakamura, Takahashi, Fukazawa, Koyanagi, Yokoyama, Kato, Yagita, & Okumura, 1990; Spits, Schooten, Keizer, Seventer, Rijn, Terhorst, & Vries, 1986). Subsequent killing of targets was likewise not impaired. This result seems contrary to the deficient killing observed in the cytolytic assays. However, the original cytolytic assays were carried out over 4 h, presumably allowing for multiple effector–target interactions with effectors recycling after initial attack to kill again (Valitutti et al., 1993). The conjugate studies, on the other hand, span 1.5 h, which perhaps is insufficient time *in vitro* for attack and recycling. Measurements made at 2.5–3 h were inconclusive due to the high background associated with propidium iodide uptake by effector and target cells.

The two groups of enriched CTL, chronic morphine-treated vs vehicle-treated, are not equivalent. One major difference is the granulation found in SL from the chronic morphine-treated animals. These results suggest that production of esterase-containing granules by the CTL subpopulation is deficient. Assuming that at the time of harvest enriched $CD8^+$ CTL from the chronic morphine- and vehicle-treated animals have both cleared the antigen stimulus, it is probable that the CTL population has recycled and awaits new targets. If at the time of harvest these CTLs represent a population "awaiting" new target then it is logical that the level of CTL granulation present represents a maximum constitutive level in the chronic morphine- and vehicle-treated groups. The 1.5-h conjugate studies of purified CTL show equivalent ability to form conjugates combined with equivalent capacity to deliver a lethal hit, but killing is impaired in the chronic morphine-treated group in the 4-h cytolytic assay results. The conjugate results, however, represent only a primary contact with target, which suggests that the level of granulation present in the awaiting CTL from chronic morphine- and vehicle-treated animals are both sufficient to deliver an initial lethal hit although their respective initial level of granulation is different. The subsequent secondary and tertiary contact with the target cells is likely to be where the defect resides. Accordingly, this points to a possible defect in CTL recycling in the chronic morphine-treated animals. The results of the cAMP studies suggest a mechanism

for this result. An increase in intracellular cAMP is associated with termination of attack after a positive interaction with target and believed to be the secondary signal that induces detachment from target, initiation of recycling, and preservation of granulation (Valitutti et al., 1993). The cAMP levels in CTLs from chronic morphine-treated animals after 30 min of antigen exposure appears reduced although basal levels in unstimulated cells are similar to that of the vehicle-treated animals. This suggests that the CTL derived from the chronic morphine-treated animals are defective in the termination of attack, and this may prolong CTL contact with target and lead to excessive degranulation and delayed recycling. Together these processes might impair subsequent killing or simply reduce the total number of lytic contacts in the 4-h time period of the cytolytic assays. In summary, two pathways of chronic morphine treatment are proposed: (1) Esterase content of CTLs is reduced. (2) Termination of attack is impaired, leading to impaired recycling and excessive degranulation and/or prolonged contact leading to a reduction in total contacts within the time frame of the assays.

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Pretreatment with β-funaltrexamine blocks morphine-mediated suppression of CTL activity in alloimmunized mice

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Abstract

The effect of prolonged exposure to morphine on cytotoxic T lymphocytes (CTL) and splenic natural killer (NK) activity was investigated. Daily administration of morphine (50.0 mg/kg, s.c.) to alloimmunized mice for 11 days resulted in a significant decrease (25–50%) in peritoneal and splenic CTL activity but not splenic NK activity. To identify the effector cell population mediating cytolysis, cell enrichment studies were carried out. The results of these studies indicated the CTLs are $CD8^+$ $CD4^-$. Chronic morphine treatment increased the percentage (25–30%) of $CD3^+CD4^+$ and $CD8^+$, but not Ig^+ cells in the spleen relative to saline-treated controls. Pretreatment of mice with the μ-selective antagonist, β-funaltrexamine blocked morphine-mediated suppression of splenic and peritoneal CTL activity as well as the increase in $CD3^+CD4^+$ and $CD8^+$ splenic lymphocytes. These results indicate the generation of CTLs in vivo is sensitive to chronic morphine exposure implicating opiates as important co-factors through modulation of cell-mediated immunity.

Keywords: Cytotoxic T lymphocyte; Morphine; Natural killer activity; β-Funaltrexamine

1. Introduction

The abuse of opioid compounds is predicted to be a major co-factor in the acquisition and spread of human immunodeficiency virus (HIV)-1 (Donahoe, 1992) due to the immunosuppressive side-effects of such drugs. Specifically, morphine has been shown to suppress picryl chloride-induced delayed-type hypersensitivity (Bryant and Roudebush, 1990), splenic NK activity (Shavit et al., 1984; Weber and Pert, 1989), primary antibody production (Pruett et al.,

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Abbreviations: BSA, bovine serum albumin; CTL, cytotoxic T lymphocyte; DMSO, dimethylsulfoxide; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; β-FNA, β-funaltrexamine; HBSS, Hanks' balanced salt solution; HIV, human immunodeficiency virus; HPA, hypothalamic-pituitary adrenal; Ig, immunoglobulin; MLC, mixed lymphocyte culture; NK, natural killer; PBS, phosphate-buffered saline; PE, phycoerythrin; PL, peritoneal exudate leukocyte(s); PWM, pokeweed mitogen; SL, splenic lymphocyte(s); TdR, thymidine deoxyribonucleic acid.

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1992; Bussiere et al., 1993) and resistance to viral infections (Lorenzo et al., 1987; Starec et al., 1991). Similarly, immunocompetence is compromised in human heroin users (Novick et al., 1989; DeShavo et al., 1989; Klimas et al., 1991) resulting in greater susceptibility to infectious agents (Dismukes et al., 1968) including HIV-1 (Hubbard et al., 1988). The acute administration of morphine suppresses NK activity through the activation of α-adrenergic pathways (Carr et al., 1993; 1994a) while chronic morphine administration activates the hypothalamic-pituitary adrenal (HPA) axis resulting in elevated levels of corticosteroids which are, in part, immunosuppressive (Bryant et al., 1991). However, the relationship between the HPA axis and other neuroendocrine pathways relative to chronic opioid-induced modulation of immune homeostasis (specifically NK and CTL activity) is still largely unknown.

In the present study, the effects of chronic administration of morphine on CTL activity in mice was investigated. Previous studies have shown the endogenous opioid peptides [Met]-enkephalin and β-endorphin augment the generation of CTLs in one-way mixed lymphocyte cultures (MLCs) in vitro through a naloxone-sensitive pathway (Carr and Klimpel, 1986). Since opioid abusers show an increased susceptibility to viral infections (Dismukes et al., 1968; Hubbard et al., 1988), an investigation assessing the immunomodulatory characteristics of morphine on CTL activity was undertaken.

2. Materials and methods

2.1. Mice and tumor lines

Female C57BL/6J (The Jackson Laboratory, Bar Harbor, ME, USA) and C3H/HeN (Harlan-Sprague Dawley, Indianapolis, IN, USA) mice were housed in groups of 6–10 per cage and maintained on a 12-h light/dark cycle. Access to water and food (Purina Mouse Chow) was available ad libitum. The YAC-1 mouse lymphoma cell line, P815 mastocytoma cell line and EL-4 lymphoma cell line were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA); the cells have been maintained in culture by biweekly passage over a 5-month period.

2.2. Morphine treatment regimen

A dose-response study has established that 50.0 mg/kg of morphine s.c. results in maximal suppression of cytolytic activity (Carr et al., 1994b). Consequently, this dose was used in all experiments.

C3H/HeN mice ($n = 8$/group) were administered the μ-opioid receptor alkylating agent β-funaltrexamine (β-FNA, 40.0 mg/kg, s.c.) or vehicle 18–24 h prior to receiving morphine (50.0 mg/kg). 2 h following morphine or vehicle administration, mice received 1×10^7 C57BL/6 spleen cells, i.p. Following the immunization, mice received vehicle or morphine daily for an additional 6 days. On day 7, mice were re-immunized with 1×10^7 C57BL/6 spleen cells, i.p. 2 h after the administration of vehicle or morphine. In addition, mice received vehicle or β-FNA (40.0 mg/kg, s.c.) every 72 h (immediately following the subsequent morphine administration) up through the 10-day incubation period. This time period corresponds to opioid receptor turnover as a result of occupancy of receptors by β-FNA (D. Paul, personal communication). On day 11, the mice were sacrificed and splenic lymphocytes (SL) and peritoneal exudate leukocytes (PL) were collected and assayed for mitogen responsiveness, CTL and NK activity. The phenotypes of the spleen cells of vehicle- and drug-treated mice were determined by flow cytometry.

2.3. SL and PL preparation

All mice were killed by CO_2 asphyxiation and peritoneal lavage was performed using 10 ml of sterile Hanks' balanced salt solution (HBSS). Cells were collected by recovery of peritoneal lavage fluid through a 20 gauge needle and 10 ml syringe. Spleens were removed and cell suspensions were prepared by mechanical dispersion. SL and PL were washed with HBSS ($250 \times g$, 5 min). Red blood cells were osmotically lysed using 0.84% NH_4Cl; the cells were subsequently washed with HBSS ($250 \times g$, 5 min) and resuspended in RPMI-1640 containing 10% fetal calf serum (FCS) and 2.5% Hybri-max (Sigma, St. Louis, MO, USA) antibiotic/antimycotic solution (complete media). Cells were counted and examined for viability using Trypan blue exclusion.

2.4. Mitogen-induced proliferation assay

SL from C3H/HeN mice (2×10^5 cells/well) in 100 μl of complete media were placed in 96-well microtiter plates (Costar, Cambridge, MA, USA). 100 μl of complete media containing 100 ng pokeweed mitogen (PWM) was added to the wells. Spleen cells were cultured in 5% CO_2 at 37°C for 48 h. After 48 h, 200 nCi [^{3}H]thymidine deoxyribonucleic acid (TdR) in HBSS (10 μl) was added to each well and the cells cultured an additional 12 h. The cells then were harvested on glass fiber filter strips using a multiple-well harvester (Cambridge Technologies, Watertown, MA, USA). Filters were placed in scintillation vials containing 6.0 ml of Cytoscint liquid scintillation cocktail (ICN, Irvine, CA, USA) and allowed to equilibrate 18–24 h. The incorporation of [^{3}H]TdR was determined by liquid scintillation counting using a Beckman LS9800. The mitogenic response of spleen cells from each animal was assayed in quadruplicate. Incorporation of [^{3}H]TdR by cells cultured in the absence of PWM was less than 5% of that obtained in maximally stimulated cultures.

2.5. ^{51}Cr-release cytolytic assay

SL and PL CTL activity was assayed using a 4-h microcytotoxicity assay with ^{51}Cr-labeled EL-4 cells (H-2^b) as targets. Between 5×10^4 and 160×10^4 effector cells were mixed with 1×10^4 target cells in conical 96-well microtiter plates (Costar) in a reaction volume of 0.2 ml of complete media. The cultures were incubated 4 h at 37°C in a 5% CO_2 atmosphere. A 100-μl aliquot of cell-free supernatant was taken from each well and its ^{51}Cr content was determined using a Beckman gamma counter. The cytolytic activity was determined as follows: percent cytolytic activity = ((experimental ^{51}Cr release – spontaneous ^{51}Cr release)/(total cell-associated ^{51}Cr release – spontaneous ^{51}Cr release)) × 100 where 'spontaneous' refers to ^{51}Cr release by target cells in the absence of effector cells. Total cell-associated ^{51}Cr was determined by measuring the ^{51}Cr content in the supernates of 10^4 target cells incubated at 37°C in a 5% CO_2 atmosphere in the presence of 0.1% *t*-octylphenoxypolyethoxyethanol in complete medium or measuring the ^{51}Cr content in 10^4 ^{51}Cr-labeled target cells. Spontaneous release was consistently between 10–15%. Each effector to target cell ratio (100:1, 50:1, 25:1 and 12:1 for SL, and 50:1, 25:1, 12:1 and 6:1 for PL) was measured in triplicate/animal. One lytic unit (LU) is defined as the number of splenic lymphocytes, peritoneal lymphocytes or enriched effector cells able to lyse 20% of the target cells (YAC-1 or EL-4 target cells) and this unit is expressed per 10^7 total cells. To determine antigen specificity for the CTL assay, P815 (H-2^d) mastocytoma cells were ^{51}Cr-labeled and used as targets in the 4-h microcytotoxicity assay.

2.6. CD4$^+$ and CD8$^+$ lymphocyte enrichment

Mouse T-cell subset enrichment column kits (R&D Systems, Minneapolis, MN, USA) were prepared as suggested by the manufacturer. SL from the saline-treated group were pooled as were SL from the morphine treatment group and separately applied to CD4 and CD8 enrichment columns. Recovered T cells were then assayed for CTL activity using ^{51}Cr-labeled EL-4 cells as targets in the ^{51}Cr release cytolytic assay.

2.7. Fluorescence-activated cell sorter (FACS) analysis of SL subpopulations

SL (1×10^6 cells/condition) obtained from the vehicle- and drug-treated groups of mice were collected and washed in 1.0 ml of phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.05 M NaN_3. SL were resuspended in 0.05 ml PBS-BSA plus NaN_3 containing 30 μg of rat IgG_{2b} (isotypic control; Zymed, South San Francisco, CA, USA) and incubated on ice for 10 min. Subsequently, antibody to CD3, CD4 and/or CD8 (rat IgG_{2b}; Gibco BRL, Gaithersburg, MD, USA) conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or α-immunoglobulin (Ig, heavy- and light-chain-specific, F(ab)$'_2$) conjugated with FITC (Boehringer-Mannheim, Indianapolis, IN, USA) was added for a final volume of 0.1 ml (using PBS-BSA plus NaN_3). The labeled cells were allowed to incubate for 30 min on ice in the dark. The cells were washed with ice-cold PBS, fixed with 1% paraformaldehyde and analyzed by

FACS for the percentage of stained cells in the cell population. Light scatter was collected at 488 nm and the emitted light which passed through a long pass filter was analyzed at 525 nm (FITC) or 575 nm (PE) on a Coulter Elite FACS (Coulter, Hialeah, FL, USA). 5000 gated events were analyzed per sample.

2.8. Reagents

Morphine sulfate and β-FNA were generously provided by the Research Technology Branch of The National Institute on Drug Abuse (Rockville, MD, USA). These drugs were initially dissolved in DMSO and diluted with HBSS to a concentration containing 10–25% DMSO. A volume of 100 μl of this solution containing the drug at the appropriate concentration was delivered to each mouse. Vehicle consisted of 10–25% DMSO in HBSS.

2.9. Statistics

One-way ANOVA (Randomized, block design) was used together with Scheffe or Tukey's post hoc multiple comparisons test to determine significance ($P<0.05$) between saline-and drug-treated groups. This statistical package used the GBSTAT program (Dynamic Microsystems, Silver Springs, MD, USA).

3. Results

3.1. Chronic morphine exposure suppresses CTL activity

Mice treated with morphine for 11 days exhibited significantly less SL CTL activity compared to vehicle-treated controls (Fig. 1). PL CTL activity was also significantly lower in the chronic morphine-treated mice compared to vehicle-treated controls (Fig. 2). However, both vehicle- and chronic morphine-treated mice showed similar levels of splenic NK activity (Fig. 3). SL from chronic morphine- and vehicle-treated mice were also assayed for lysis of a histoincompatible target. Neither population of SL showed any measurable cytolytic activity against ^{51}Cr-labeled P815 cells (data not shown). In addition, SL from unprimed (non-immunized) mice had no measurable CTL activity to the ^{51}Cr-labeled EL-4 targets (data not shown).

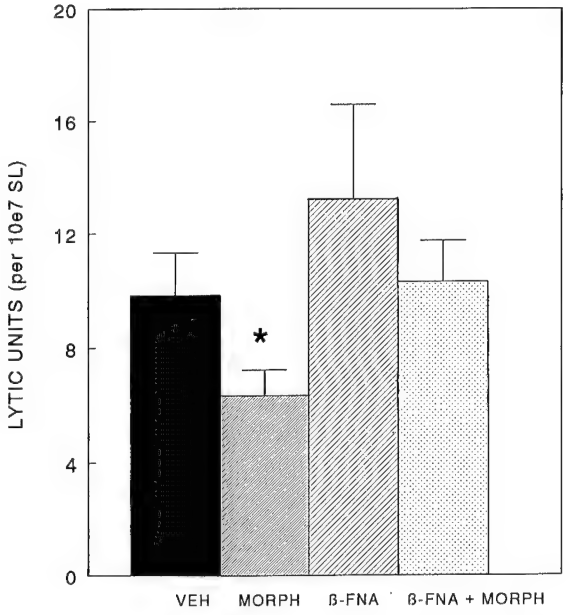

Fig. 1. β-FNA reverses morphine-induced suppression in splenic lymphocytes CTL activity. C3H/HeN mice ($n=8$/group) were administered β-FNA (40.0 mg/kg, s.c.) or vehicle 18–24 h prior to receiving morphine (50.0 mg/kg, s.c.) followed by alloimmunization (1×10^7 C57BL/6J splenocytes, i.p.) 2 h later. Mice received morphine (50.0 mg/kg, s.c.) or vehicle daily for the next 10 days. In addition, mice received β-FNA (40.0 mg/kg, s.c.) every 72 h. Mice were re-immunized (1×10^7 C57BL/6J splenocytes, i.p.) 6 days following the initial immunization. The animals were killed on day 11 and their splenic lymphocytes (SL) assayed for CTL activity using ^{51}Cr-labeled EL-4 cells. Bars represent SEM, $n=8$. *$F(3,31)=1.9646$, $P=0.05$ comparing vehicle- to chronic morphine-treated group as determined by ANOVA and Tukey's multiple comparison test.

Using cell separation techniques, the SL CTL derived from vehicle-and drug-treated mice (Fig. 4) were found to be CD8$^+$ (Table 1). CD8$^+$-enriched effector cells were also tested against the NK-sensitive target YAC-1 and found not to lyse these cells, indicating the antigen-specificity of the enriched effector cells (data not shown). Moreover, enrichment enhanced the difference in CTL activity between SL obtained from vehicle- and chronic morphine-treated mice (Table 1).

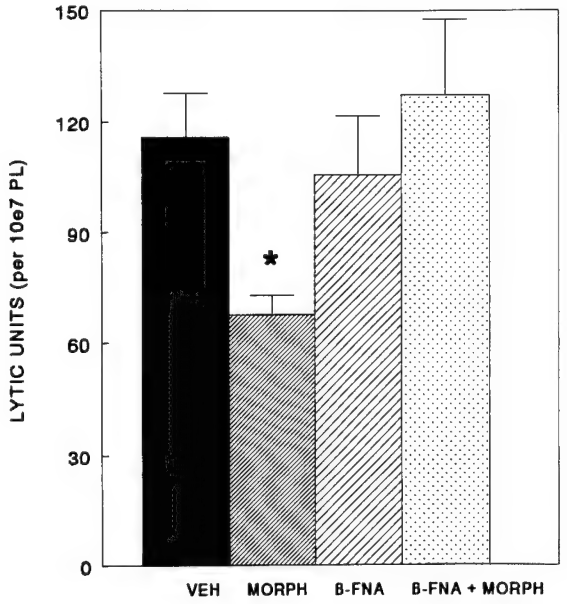

Fig. 2. Long-acting μ-antagonist β-FNA blocks morphine-induced suppression of CTL activity of peritoneal lymphocytes. Mice were treated as described in the legend to Fig. 1. Peritoneal lymphocytes (PL) were collected and assayed for CTL activity using ^{51}Cr-labeled EL-4 cells. PL CTL activity from chronical morphine-treated mice was significantly suppressed relative to other treatment groups. β-FNA co-treatment completely reversed this effect. $*F(3.18) = 3.9754$, $P < 0.05$ comparing chronic morphine-treated animals to all other groups of mice as determined by ANOVA followed by Scheffé multiple comparison test. Bars represent SEM, $n = 7$.

Table 1

Splenic CTL effector cells are CD8$^+$ [a]

Treatment	Unfractionated	CD4$^+$-enriched	CD8$^+$-enriched
Vehicle	17.7 ± 1.9[b]	1.8 ± 1.4	74.0 ± 6.4
Morphine	8.0 ± 1.6	1.6 ± 1.5	15.2 ± 5.3

[a] SL from vehicle- and chronic morphine-treated mice ($n = 3$/group) were enriched for either CD4$^+$ or CD8$^+$ cells (see Materials and Methods). Prior to enrichment, SL were assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. Cells from each treatment group were pooled and subsequently enriched for CD4$^+$ or CD8$^+$ cells, which subsequently were assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. Between 80–90% of SL were lost as a result of the enrichment step which is consistent with the fact that SL contain 8–12% CD8$^+$ cells (Fig. 4A). This table is a summary of two independent experiments with similar outcomes.

[b] Numbers are in LU ± SEM, $n = 3$.

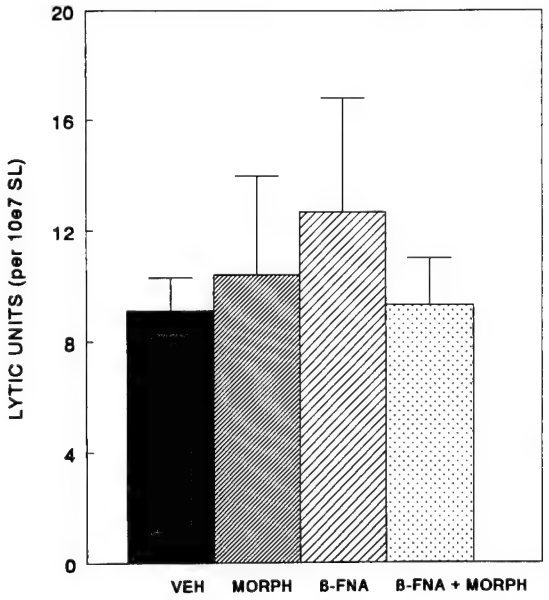

Fig. 3. β-FNA alone or in combination with morphine had no effect on splenic NK activity. Mice were treated as described in the legend to Fig. 1. Splenic lymphocytes (SL) were collected and assayed for NK activity using ^{51}Cr-labeled YAC-1 cells. Bars represent SEM, $n = 8$.

3.2. β-FNA attenuates opioid-induced suppression of SL and PL CTL activity in chronic morphine-treated mice

To determine opioid receptor involvement in morphine-mediated suppression of CTL activity, studies were carried out using the μ-selective opioid antagonist β-FNA in morphine-treated animals. Pretreatment of mice with β-FNA completely blocked morphine-induced suppression of SL (Fig. 1) and PL (Fig. 2) CTL activity. β-FNA alone had no effect on SL (Fig. 1) or PL (Fig. 2) CTL activity. β-FNA alone nor in combination with morphine had any effect on splenic NK activity (Fig. 3).

3.3. β-FNA blocks morphine-induced increases in the percentage of CD4$^+$ and CD8$^+$ splenic lymphocytes

Since morphine decreased the SL and PL CTL activity and splenic effector cells mediating antigen-specific CTL activity were defined as CD4$^-$CD8$^+$, morphine exposure might modify the number of

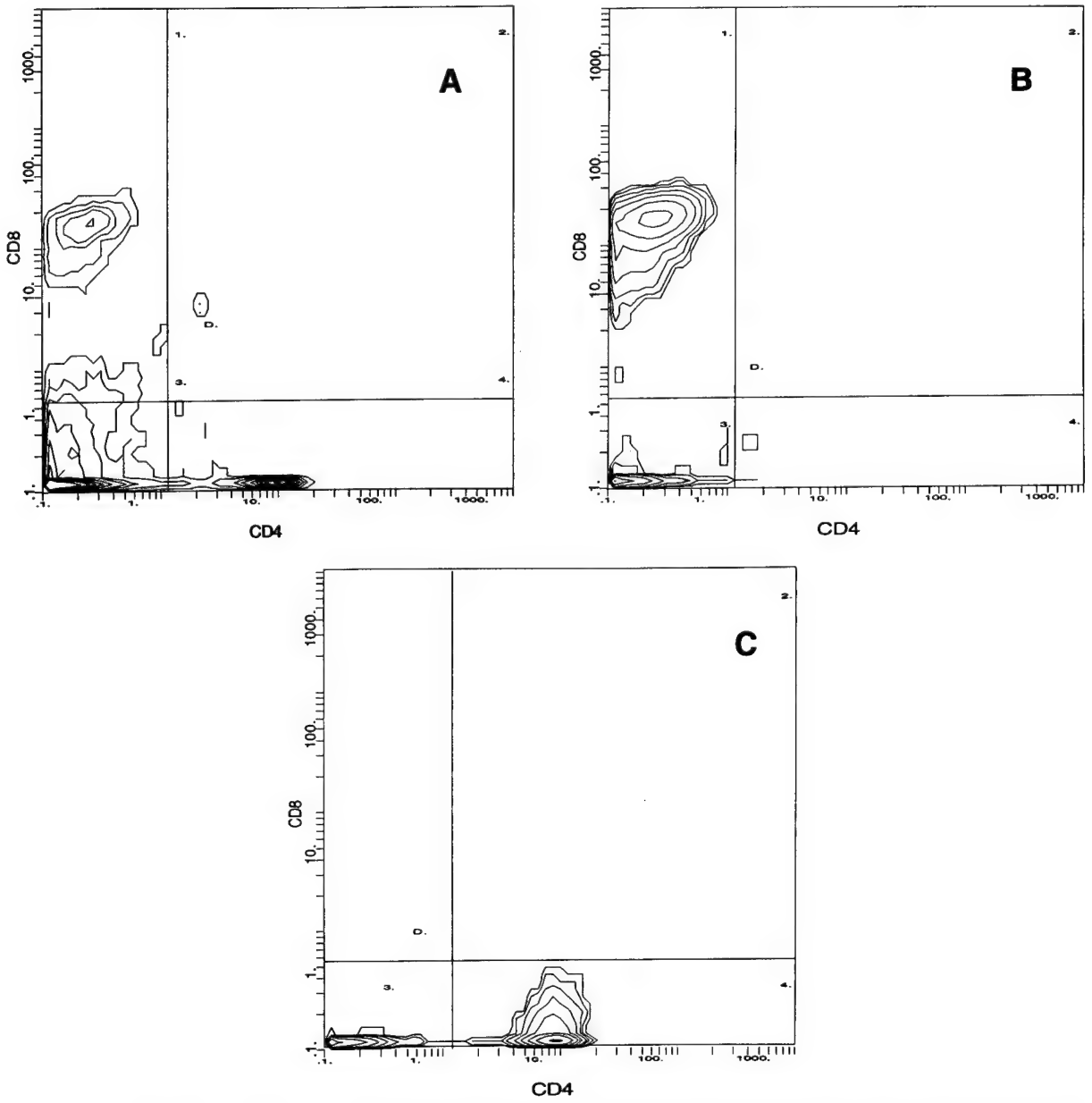

Fig. 4. Cytometric analysis of $CD4^+$- and $CD8^+$-enriched splenic lymphocytes. Splenic lymphocytes obtained from mice treated as described in the legend to Fig. 1 were labelled using anti-CD4 antibody conjugated to FITC and anti-CD8 antibody conjugated to PE. (A) Splenocytes prior to enrichment. (B) Splenocytes following $CD8^+$ enrichment. (C) Splenocytes following $CD4^+$ enrichment.

lymphocytes in the spleen. Studies were undertaken to assess T_{helper} ($CD3^+CD4^+$), $T_{cytotoxic}$ ($CD8^+$) and B (Ig^+) splenic and peritoneal lymphocyte populations from vehicle and chronic morphine-treated mice in the presence and absence of β-FNA. Phenotypic analysis of SL populations revealed an increase in the percentage of $CD3^+CD4^+$ and $CD8^+$ but not Ig^+ cells in the chronic morphine-treated mice (Fig. 5). Pretreatment of mice with β-FNA blocked the effects of morphine on the

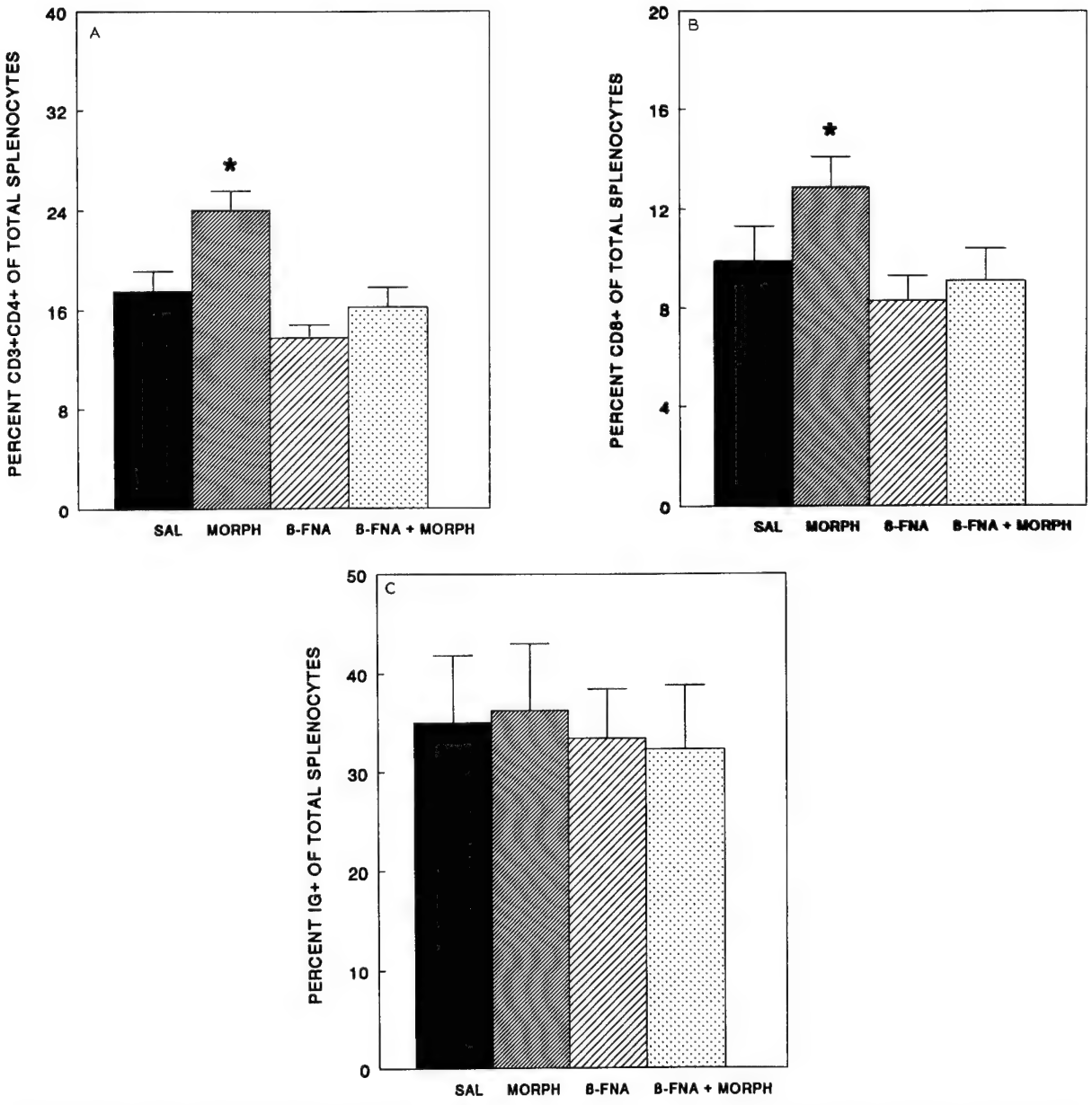

Fig. 5. β-FNA antagonizes morphine-mediated increases in splenic $CD3^+CD4^+$ and $CD8^+$ subpopulations. Mice were treated as described in the figure legend to Fig. 1. (A) β-FNA antagonized the increase in the percentage of $CD3^+CD4^+$ splenic lymphocyte following chronic morphine administration. $*F(1,6) = 4.9923$, $P < 0.05$ comparing vehicle- to chronic morphine-treated mice as determined by ANOVA and Scheffé multiple comparison test. All other groups were not significant compared to vehicle-treated animals. Bars represent SEM, $n = 7$. (B) β-FNA antagonized the increase in the percentage of $CD8^+$ splenic lymphocytes following chronic morphine administration. $*F(3,15) = 7.4202$, $P < 0.05$ comparing chronic morphine-treated mice to all other groups as determined by ANOVA and Tukey's t-test). Bars represent SEM, $n = 6$. (C) Chronic morphine exposure had no effect on the percentage of Ig^+ splenic lymphocytes. Bars represent SEM, $n = 7$.

percentage shifts in the SL population (Fig. 5) although β-FNA alone had no effect.

Changes in the percentage of lymphocyte populations in the spleen may also alter lymphocyte

Table 2

β-FNA partially antagonizes morphine-mediated augmentation of the SL proliferative response to PWM[a]

Treatment	Counts per minute ± SEM
Vehicle	14542 ± 588
Morphine	20869 ± 611**
β-FNA	13715 ± 83
Morphine + β-FNA	17622 ± 644**,*

[a] SL obtained from mice (n = 8/group) treated as described in the legend to Fig. 5 were cultured in the presence of PWM for 48 h. 200 nCi of [^{3}H]TdR was added to the wells and cells were incubated an additional 12 h. Cells were harvested and assayed for [^{3}H]TdR incorporation.

* $P<0.05$ comparing SL from mice co-administered β-FNA + morphine to chronic morphine-treated animals as determined by ANOVA and Scheffé multiple comparison test.

** $F(3,21) = 63.1663$, $P<0.01$ comparing SL from chronic morphine-treated mice to vehicle-treated controls as determined by ANOVA and Scheffé multiple comparison test.

responsivity to antigen (as shown in the CTL activity) or mitogen through the absence of appropriate cytokines necessary to drive lymphocyte growth and differentiation. Accordingly, SL from the treated groups of animals were also evaluated for proliferation to PWM. SL from chronic morphine-treated mice showed a significant increase in response to PWM compared to splenocytes from saline-treated controls (Table 2). Pretreatment of mice with β-FNA partially antagonized the effect of morphine. Pretreatment with β-FNA alone had no appreciable effect on PWM-stimulated SL proliferation (Table 2).

4. Discussion

In the present study, chronic exposure to morphine resulted in a lower response to alloimmunization as reflected by CTL activity. Since the reduced CTL activity was evident in both PL and SL populations, we speculated that a systemic pathway is involved. The results of previous studies indicate that 72-h exposure to morphine activates the HPA axis resulting in the elevation in serum corticosterone levels (Bryant et al., 1991). Endogenous corticosterone is selectively immunosuppressive (Stein and Miller, 1993) and may be partly responsible for the effects seen in the present study. However, recent data may indicate otherwise. Specifically, corticosterone levels have been measured in the chronic (11 day) morphine-treated mice following the killing of the animals and found to be reduced in comparison to the levels from vehicle-treated mice (Carpenter et al., 1994). However, this observation does not rule out the role elevated levels of corticosterone may have earlier in the immune response as indicated by others (Bryant et al. 1987; 1990; 1991; Fuchs and Pruett, 1993).

Adrenergic pathways have also previously been shown to be involved in morphine-induced immunosuppression of SL (Carr et al., 1993, Fecho et al., 1993). Interestingly, the immunosuppression following acute morphine administration is compartment specific (Bayer et al., 1990; Baddley et. al., 1993; Lysle et al., 1993). The compartmentalized nature of morphine-mediated immunomodulation may in part lie with the neuroendocrine systems innervating the immune organ (Felten et al., 1985), as well as the state of activation of the lymphocytes. Specifically, although previous work indicates lymphocytes possess μ-type opioid binding sites (Madden et al., 1987; Radulescu et al., 1991), recent studies indicate activation upregulates the expression of the morphine-sensitive binding site (Roy et al., 1992). However, morphine (10^{-5}–10^{-11} M) effects on the generation of CTLs in one-way MLCs in vitro have resulted in no discernable differences to vehicle-treated controls (unpublished observation); this observation indicates the absence of a direct effect of morphine on lymphocytes relative to CTL activity. Moreover, a previous study showed morphine-mediated immunoregulation did not correlate with circulating levels of the drug (Bryant et al., 1988). Collectively, these results suggest morphine-mediated immunomodulation following acute or chronic application of drug in vivo acts in part through neuroendocrine pathways other than the HPA axis as most recently illustrated (Hernandez et al., 1993; Carr et al., 1994a).

The pretreatment of mice with the irreversible μ-selective opioid receptor antagonist, β-FNA (Ward et al., 1982), effectively blocked the suppression of PL and SL CTL activity precipitated by chronic morphine treatment. Similar findings have also been reported for splenic NK activity following

acute morphine administration (Band et al., 1992; Carr et al., 1993). The selected dose of β-FNA was chosen based on previous data showing pretreatment of mice with β-FNA blocks [D-Ala^2, ME-Phe^4, gly(ol)^5]enkephalin- but not [D-Pen^2, D-Pen^5]enkephalin-induced analgesia in mice (Paul et al., 1989). Another recent study has shown the δ-selective opioid receptor antagonist (E)-7-benzylidine-7-dihydronaltrexone (BNTX; Porteghese et al., 1992) does not block morphine-mediated suppression of SL or PL CTL activity (Carr and Carpenter, data not shown), suggesting μ- but not δ-opioid receptor involvement.

Morphine-mediated suppression of CTL activity in alloimmunized mice may be due to immune dysfunction at the cellular level. Serine esterases, such as the BLT esterase, have been identified in CTLs (Pasternack and Eisen, 1985), are released after specific target cell binding (Pasternack et al., 1986), and have been localized to the cytotoxic granules (Young et al., 1986) implicating these enzymes as a mechanism of target cell lysis by CTLs. Other studies have indicated that serine esterases are not necessary for target cell lysis depending on the target (Trenn et al., 1987; Ostergaard et al., 1987). By inhibiting serine esterase release from CTL clones using cyclosporine A, another laboratory has hypothesized the existence of a cyclosporine-sensitive capacity to induce target cell lysis and a cyclosporine-insensitive mechanism of inducing lysis of target cells that does not require granule exocytosis (Lancki et al., 1989). Recently, a third mechanism of target cell lysis by CD4$^+$ and CD8$^+$ T cells has been identified which predominately involves direct TNF-α-dependent lysis of TNF-α-sensitive targets (Smyth and Ortaldo, 1993). A recent study showed serine esterase release and total cell serine esterase content was reduced in SL taken from chronic morphine-treated mice compared to vehicle-treated controls (Carpenter et al., 1994). In addition, no differences were found in the number of SL conjugating with targets, suggesting the conjugating process of CTLs generated over the course of 11 days was similar between vehicle- and chronic morphine-treated animals (Carpenter et al., 1994). The suppression of serine esterase release and total serine esterase content of lymphocytes from chronic morphine-treated animals is consistent with a role for this enzyme in lysing target cells. Similar results have also been obtained using IL-2- and IL-12-stimulated human CD8$^+$ T cells (Mehrotra et al., 1993). In support of this hypothesis, SL CTLs from vehicle- and chronic morphine-treated mice lyse the IL-4 targets through a Ca^{2+}-dependent process (Carr and Carpenter, data not shown) implicating granzyme A (contains serine esterases) in the 'lethal hit' (Berke, 1994). Consequently, one explanation for the reduction in cytolytic capacity of lymphocytes chronically exposed to morphine in vivo is a reduced capacity to produce granules which contain serine esterases and/or an inability to exocytosis esterase-containing granules.

In the present study, chronic morphine exposure was found to elevate the percentages of both CD4$^+$ and CD8$^+$ cells in the spleen. SL Ig$^+$ cell numbers were not affected. These results conflict with previous data showing a time-dependent increase in the CD4$^+$ cells and decrease in the CD8$^+$ cells in the spleen (Arora et al., 1990). The results of another study revealed a decrease in both CD4$^+$ and CD8$^+$ cells (Kimes et al., 1992). The discrepancies in the results may be due to the time course of morphine treatment. In the present study, morphine administration was continued for 11 days while the other studies employed morphine pellet implants and measured T-cell subsets 72–120-h post implantation (Arora et al., 1990; Kimes et al., 1992). Another difference between these investigations involves the different strains of mice employed. It has previously been shown that there are strain differences in susceptibility to morphine effects on immune responses (Bussiere et al., 1992). Our results indicate that chronic morphine treatment increases the numbers of both CD4$^+$ and CD8$^+$ cells in the spleen. Furthermore, there is an increase in proliferation in response to PWM by SL from chronic morphine-treated mice compared to SL from vehicle-treated controls which is opposite to reports assessing mitogen-induced lymphocyte proliferation following acute morphine administration (Hernandez et al., 1994). The response to mitogen by SL from chronic morphine-treated animals may reflect changes in the population of T_H subpopulations. Additional studies using CD markers expressed on these cells are in progress. Other studies using chronic morphine-treated rhesus monkeys has shown an increase in the percentage of CD4$^+$ CD29$^+$ PBMCs and a decrease

the percentage of $CD4^+CD45RA^+$ PBMCs (Carr and France, 1993). Such a shift in the memory/helper $CD4^+$ population may alter cytokine production affecting the generation of CTLs.

The data indicating that chronic morphine exposure acts selectively on antigen-driven cytolytic function and not NK cytolytic activity may implicate morphine in the regulation of cytokine production. Since CTL maturation and maintenance of function require IL-2 and in certain instances IL-6 (Bass et al., 1993), morphine regulation of the production of these cytokines could affect CTL generation and activity. In support of this hypothesis, it is known that morphine-mediated suppression of the primary antibody response is the result of a reduction in IL-6 synthesis; exogenous IL-6 attenuated the suppression in antibody production (Bussiere et al., 1993). Another study revealed that morphine pellet implantation has no effect on PMA-induced IL-2 production (Saini and Sei, 1993); it may be that morphine selectively inhibits specific cytokines. Whereas morphine may reduce the production of some cytokines, recent data indicate that it augments TGF-β production in vitro (Chao et al., 1992); this cytokine is a negative regulator of many T-cell responses (Ishizaka et al., 1992).

In summary, the data indicate that prolonged exposure to morphine diminishes the capacity to sustain CTL activity to alloantigenic cells. Since morphine (and heroin) has previously been shown to promote the growth of HIV-1 in mitogen-stimulated PBMCs in vitro (Peterson et al., 1990; Adler et al., 1993) and both reduce immune responses, the molecular mechanisms of action of opioids in affecting immune homeostasis will be a primary focus of future research.

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Morphine-Induced Suppression of Cytotoxic T Lymphocyte Activity in Alloimmunized Mice Is Not Mediated through a Naltrindole-Sensitive Delta Opioid Receptor

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Key Words
Morphine
Cytotoxic T lymphocyte
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Natural killer activity

Abstract
The effect of chronic morphine exposure on natural killer (NK) activity in vivo and the generation of cytotoxic T lymphocytes (CTLs) in vitro and in vivo was investigated. Chronic exposure to morphine (10^{-5}–10^{-11} *M*) in vitro had no effect on the generation of antigen-driven effector cells. However, the daily administration of morphine (50.0 mg/kg, s.c.) into alloimmunized mice (C57BL/6 into C3H/HeN) for 11 days resulted in a decrease in peritoneal and splenic CTL activity but not splenic NK activity. In addition, there was a 60% decrease in the number of thymocytes recovered from chronic morphine-treated mice compared to vehicle-treated controls. However, the overall percentage of CD4+CD8–, CD4–CD8+ and CD4+CD8+ thymocytes did not change between the two groups of treated animals. Pretreatment of the mice with the δ_1-selective antagonist, (E)-7-benzylidine-7-dihydronaltrexone (BNTX, 0.6 mg/kg, s.c.) did not block morphine-mediated suppression of splenic CTL activity but did block morphine-induced suppression of peritoneal lymphocyte CTL activity. In addition, BNTX pretreatment alone augmented splenic NK activity and such augmentation was blocked following chronic morphine exposure. In contrast, the δ-selective antagonist, naltrindole (20.0 mg/kg, s.c.), had no effect alone nor antagonized the action of morphine on CTL activity. Splenic CTL effector cells from either treated group of animals lysed their target (EL-4 lymphoma) through a Ca^{2+}-dependent mechanism. Collectively, the results indicate morphine suppresses CTL activity through an indirect pathway, insensitive to naltrindole rather than through direct lymphocyte opioid receptors.

Introduction

The abuse of opioids (e.g. heroin and fentanyl) resulting in a compromised immune system [1–3] and a greater susceptibility to infectious agents [4, 5] has made this class of compounds a liability and predicted cofactor in the acquisition and spread of HIV-1 [6]. The implicated cofactor relationship between opioids and HIV-1 is supported by data showing morphine amplification of HIV-1 expression in phytohemagglutinin-activated peripheral

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blood mononuclear cells [7]. In addition, morphine increases lipopolysaccharide-primed microglial production of tumor necrosis factor-α which in turn promotes HIV-1 expression in latently infected promonocytes [8] and promonocyte-fetal brain cell cocultures [9]. Within the immune system, chronic morphine treatment has been shown to increase the percentage of CD4+CD29+ peripheral blood T lymphocytes in rhesus monkeys [10] which are the reported reservoirs for simian immunodeficiency virus [11]. At the molecular level, pokeweed-mitogen-stimulated peripheral blood mononuclear cells from rhesus monkeys treated chronically with morphine possess elevated levels of NFκB which is a regulatory element for key cytokines including interleukin-2 and tumor necrosis factor-α and binds to sites within the HIV-1 promoter [12]. Taken together, the data would suggest opioids increase transcriptional regulators which promote HIV-1 replication, augment the reservoir cell population for HIV-1 replication in the peripheral blood and increase cytokine production by microglial cells in the brain increasing the likelihood of replication of this virus in the central nervous system. However, the effector cell population (cytotoxic T lymphocytes, CTL) which is typically responsible for monitoring virus infection has only recently been evaluated in the presence of opioids.

Previous studies have shown the endogenous opioid peptides β-endorphin and [met]-enkephalin augment the generation of CTLs in one-way mixed lymphocyte cultures (MLCs) [13]. More recently, alloimmunized mice chronically treated with morphine (50.0 mg/kg, s.c., daily for 11 days) presented with significantly suppressed CTL activity in splenic and peritoneal lymphocyte populations [14]. Moreover, the μ-selective opioid receptor antagonist, β-funaltrexamine, blocked the suppression of CTL activity in the chronic morphine-treated mice, suggesting the effect was mediated in part by μ opioid receptors [15]. The mechanism of this suppression was identified to include a decrease in the production and release of serine esterases which are typically associated with the 'lethal hit' by a proportion but not all CTLs [14]. The release of granules (granzyme A) containing the serine esterases is a Ca^{2+}-dependent phenomenon while the other mode of CTL-directed lysis of target cells involves receptor-mediated apoptosis which does not require extracellular Ca^{2+} [16].

Therefore, we investigated the initial observations of morphine-mediated suppression of CTL activity: (i) the potential involvement of δ opioid receptors; (ii) the calcium-dependent nature of CTL-directed cytolysis, and (iii) T cell precursor development through the assessment of thymic subpopulations in chronic morphine-treated mice.

Materials and Methods

Mice and Tumor Lines

Female C57BL/6 and C3H/HeN (Harlan Sprague Dawley, Indianapolis, Ind., USA) mice (6–7 weeks of age) were housed in groups of 5 per cage and maintained on a 12-hour light/dark cycle. Access to water and food (Purina Mouse Chow) was available ad libitum. The YAC-1 and EL-4 mouse lymphoma cell lines and P815 mastocytoma cell line were originally obtained from the American Type Culture Collection (Rockville, Md., USA); the cells have been maintained in culture by biweekly passage over a 6-month period. All animals used in these studies were maintained in accordance with the Committee on the Use and Care of Animals, Louisiana State University Medical Center, and the guidelines of the Committee on Care and Use of Laboratory Animals Resources, National Research Council, Department of Health, Education, and Welfare Publications Number (National Institutes of Health) 85-23 revised 1985.

Morphine Treatment Regimen

A dose-response study has established that 50.0 mg/kg of morphine s.c. result in maximal suppression of cytolytic activity [17; unpubl. observation]. Consequently, this dose was used in the in vivo experiments.

C3H/HeN mice (n = 10/group) were administered vehicle or the δ opioid receptor antagonists BNTX (0.6 mg/kg, s.c.) or naltrindole (20.0 mg/kg, s.c.) 30 min prior to the initiation of the chronic morphine treatment. Morphine was administered 2 h prior to receiving 1×10^7 C57BL/6 spleen cells, i.p. Following the immunization, mice received vehicle or morphine daily for an additional 6 days. On day 7, mice were reimmunized with 1×10^7 C57BL/6 spleen cells, i.p., 2 h after the administration of vehicle or morphine. Following the second immunization, mice received morphine or vehicle daily for an additional 3 days. In addition to the daily administration of morphine or vehicle, mice received either vehicle, naltrindole (20.0 mg/kg, s.c.) or BNTX (0.6 mg/kg, s.c.) daily 30 min prior to morphine administration. On day 11 after the initial immunization, the mice were sacrificed and peritoneal (PL) and splenic lymphocytes (SL) were assayed for CTL and NK activity. The concentration of BNTX used in this study was found to be fully antagonistic to [(D-Pen^2, D-Pen^5)enkephalin] in mice [18]. Likewise, the concentration of naltrindole used in this study has previously been shown to be selective for antagonizing δ but not μ or κ opioid receptors [19]. In addition, splenic lymphocytes generated in this manner have previously been shown to be antigen-specific, CD8+CD4– effector cells [14, 15].

Lymphocyte and Thymocyte Preparation

All mice were sacrificed by CO_2 asphyxiation and peritoneal lavage was performed using 10 ml of sterile Hanks' balanced salt solution (HBSS). Cells were collected by recovering 10 ml of peritoneal fluid through a 20-gauge needle and 10-ml syringe. Thymus and spleens were removed and cell suspensions were prepared by mechanical dispersion. SL, PL and thymocytes were washed with HBSS (250 *g*, 5 min). Red blood cells were osmotically lysed using 0.84% NH_4Cl; the cells were subsequently washed with HBSS (250 *g*, 5 min)

and resuspended in RPMI-1640 containing 10% fetal calf serum and 2.5% Hybri-max (Sigma, St. Louis, Mo., USA) antibiotic/antimycotic solution (complete media). Cells were counted and examined for viability via trypan blue exclusion dye.

In vitro Generation of Cytotoxic Effector Cells

Sterile suspensions of SL were prepared, and red blood cells were osmotically lysed as described above. In vitro cultures were established in sterile flat-bottomed 24-well microtiter plates (Costar, Cambridge, Mass., USA) with complete media. C3H/HeN SL (6×10^6 cells) were cocultured for 72–120 h with irradiated (900 rad) stimulator (C57BL/6) cells (4×10^6 cells) in the presence or absence of morphine (10^{-5}–10^{-11} *M*). The indicated amount of morphine was added daily to the cultures. These cultures were incubated in 5% CO_2 atmosphere at 37 °C. At the end of the culture period, cells were pelleted (200 *g*, 5 min), washed with HBSS and resuspended in a volume of complete media to give the effector-to-target cell ratios of 40:1, 20:1, 10:1 and 5:1. Each effector-to-target cell ratio was determined in triplicate. The percent killing at each effector-to-target ratio was converted to lytic units (LU). One LU was defined as the number of effector cells able to lyse 30% of the targets (EL-4 lymphomas) and this unit was expressed per 10^7 cells.

^{51}Cr-Release Cytolytic Assay

SL and PL CTL activity was assayed using a 4-hour microcytotoxicity assay with ^{51}Cr-labeled EL-4 cells (H-2^b) or P815 cells (H-2^d) as targets. ^{51}Cr-labeled YAC-1 lymphoma cells were used as targets to measure SL NK activity. Between 2×10^4 and 100×10^4 effector cells were mixed with 1×10^4 target cells in conical 96-well microtiter plates (Costar) in a reaction volume of 0.2 ml of complete media. The cultures were incubated 4 h at 37 °C in a 5% CO_2 atmosphere. A 100-µl aliquot of cell-free supernate was taken from each well and its ^{51}Cr content was determined using a Beckman γ-counter. The cytolytic activity was determined as follows: percent cytolytic activity = [(experimental ^{51}Cr release – spontaneous ^{51}Cr release)/(total cell-associated ^{51}Cr release – spontaneous ^{51}Cr release)] × 100, where 'spontaneous' refers to ^{51}Cr release by target cells in the absence of effector cells. Total cell-associated ^{51}Cr was determined by measuring the ^{51}Cr in the supernate of target cells lysed with 0.1% Triton X-100 in complete medium. Each effector-to-target cell ratio was measured in triplicate/animal. The percent lysis was then converted to LU. One LU was defined as the number of SL or PL able to lyse 20% of the target cells, and this unit was expressed per 10^7 cells. To determine antigen specificity for the in vitro generated CTL assay, P815 mastocytoma cells were ^{51}Cr-labeled and used as targets in the 4-hour microcytotoxicity assay. To determine the Ca^{2+} requirement in the CTL-directed cytolysis of the target cell, SL CTLs were incubated in the presence or absence of 3.6 m*M* EGTA during the 4-hour microcytotoxicity assay.

FACS Analysis of Thymocyte Populations

Thymocytes from the vehicle- or drug-treated groups of mice were collected and washed in RDF buffer (R & D Systems, Minneapolis, Minn., USA) and resuspended in 20 µl of RDF buffer containing fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 (IgG2b isotype, Gibco BRL, Gaithersburg, Md., USA) and phycoerythrin (PE)-conjugated rat anti-mouse CD8 (IgG2b isotype, Gibco BRL). FITC- and PE-conjugated rat IgG2b were used as isotypic controls. The cells were allowed to incubate for 20–25 min on ice in the dark. 900 µl RDF buffer were added to the cells which were then centrifuged (250 *g*, 5 min). The supernatant fluid was discarded, and the cell pellet was resuspended in 250 µl of RDF buffer and 250 µl of 2% paraformaldehyde (Sigma) and analyzed by FACS for the percentage of stained cells in the cell population. Light scatter was collected at 488 nm, and the emitted light which passed through a long pass filter was analyzed at 525 nm (FITC) or 575 nm (PE) on a Coulter Elite FACS (Coulter, Hialeah, Fla., USA). 5,000 gated events were collected and analyzed per sample. Compensation between FITC and PE amounted to 20–25%.

Reagents

Morphine sulfate was generously provided by the Research Technology Branch of the National Institute on Drug Abuse (Rockville, Md., USA). The δ_1-selective opioid receptor antagonist BNTX and naltrindole were purchased from Research Biochemicals (RBI, Natick, Mass., USA). The drugs were dissolved in 10% dimethyl sulfoxide in HBSS. Vehicle consisted of 10% dimethyl sulfoxide in HBSS.

Statistics

One-way ANOVA (randomized, block design) was used together with Tukey's protected t test or Scheffé's multiple comparison tests in comparing individual means between treated groups of animals in order to determine significance ($p < 0.05$). This statistical package used the GBSTAT program (Dynamic Microsystems, Silver Springs, Md., USA).

Results

Morphine Exposure in vitro Has No Effect on the Generation of Antigen-Driven Effector Cells

One-way MLCs were set up to determine the direct effects of morphine on the generation of cytolytic effector cells. The results show morphine (10^{-5}–10^{-11} *M*) added to cultures daily had no effect on the production of cytolytic effector cells compared to vehicle-treated controls determined 72, 96 and 120 h following initiation of culture (fig. 1). Cytolytic effector cells collected on day 5 following culture were also tested for target specificity using ^{51}Cr-labeled P815 mastocytoma cells (H-2^d haplotype). No measurable cytolysis of this cell line was detected (data not shown).

Chronic Morphine Treatment Antagonizes the BNTX-Mediated Augmentation of Splenic NK Activity

C3H/HeN mice chronically administered morphine exhibited lower SL (fig. 2) and PL CTL (fig. 3) activity compared to vehicle-treated controls. Consistent with previous results, chronic morphine exposure had no effect on splenic NK activity (fig. 4). Pretreatment with BNTX antagonized morphine-induced suppression of PL CTL activity (fig. 3) but not SL CTL activity (fig. 2). Pretreatment of mice with BNTX alone had no effect on SL

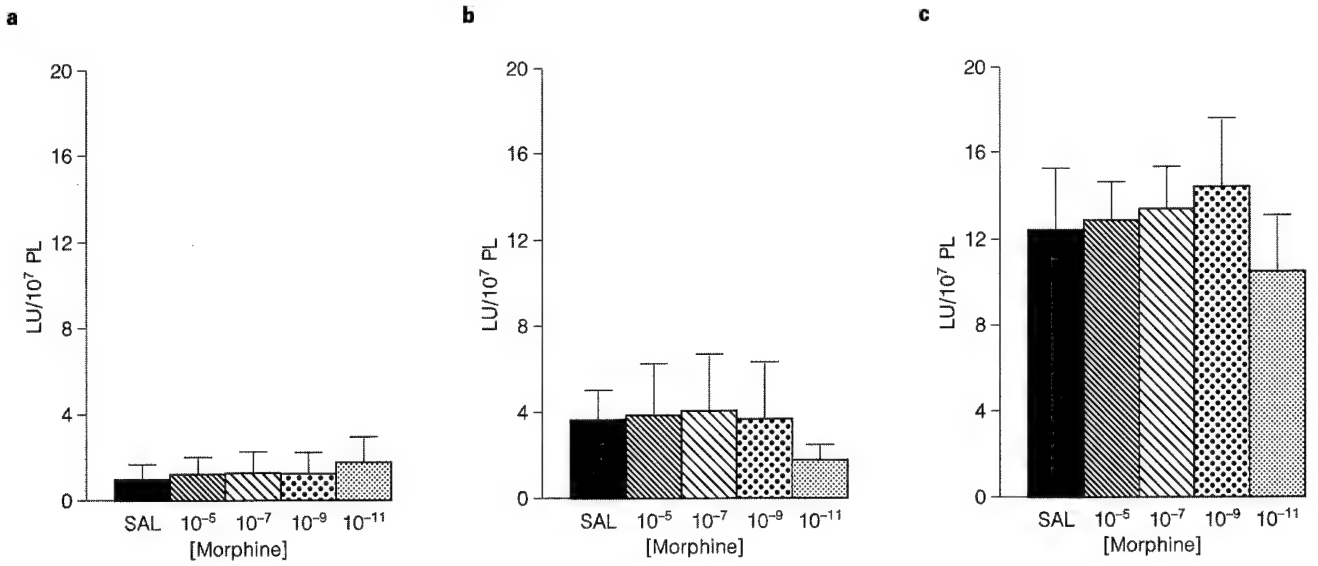


Fig. 1. Chronic morphine exposure has no direct effect on the generation of antigen-driven effector cells in one-way MLC. C3H/HeN SL (6×10^6 cells) were cocultured with irradiated (900 rad) C57BL/6 SL (4×10^6 cells) in the presence or absence of the indicated concentration of morphine added daily to each culture. Vehicle was added to control cultures. Following 72- (**a**), 96- (**b**) or 120-hour (**c**) culture incubation periods, the cells were harvested, enumerated and assayed for cytolytic activity using ^{51}Cr-labeled EL-4 cells as targets. Bars represent SEM, n = 5. SAL = Saline.

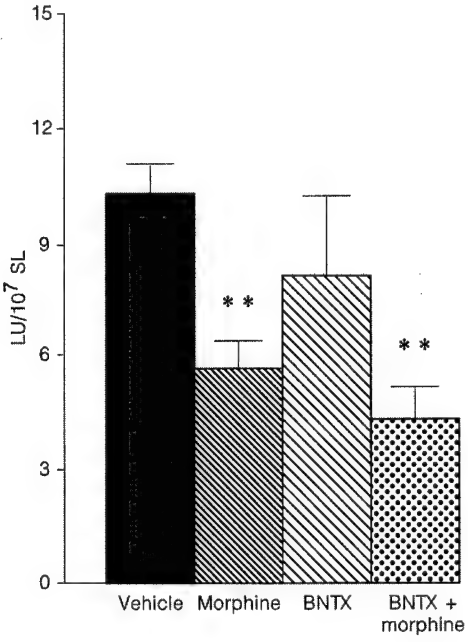
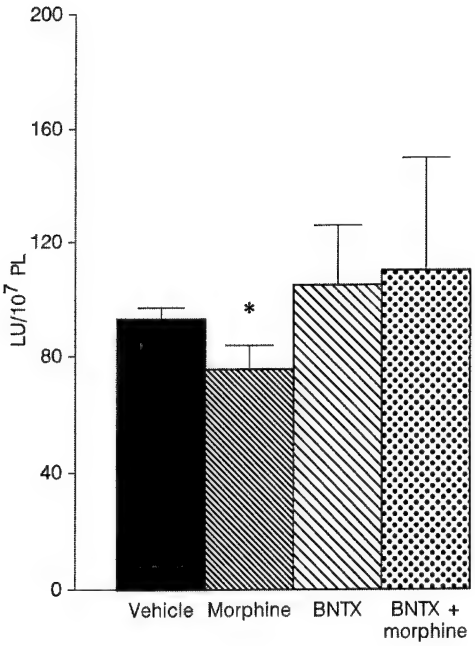


Fig. 2. BNTX does not antagonize morphine-mediated suppression of splenic CTL activity. C3H/HeN mice (n = 9/group) were administered BNTX (0.6 mg/kg, s.c.) or vehicle 30 min prior to receiving morphine (50.0 mg/kg, s.c.) or vehicle. Two hours after morphine administration, mice were alloimmunized (1×10^7 C57BL/6 splenocytes, i.p.). Groups of mice received morphine (50.0 mg/kg, s.c.), BNTX (0.6 mg/kg, s.c.), both morphine and BNTX, or vehicle daily for 10 additional days. All mice were reimmunized (1×10^7 C57BL/6 splenocytes, i.p.) 6 days after the initial immunization. Animals were sacrificed on day 11 and SL were collected and assayed for CTL activity using ^{51}Cr-labeled EL-4 cells. Chronic morphine or BNTX + morphine exposure significantly suppressed splenic CTL activity, ** $p < 0.01$ comparing drug-treated to vehicle controls as determined by ANOVA and Tukey's post t test. Bars represent SEM.



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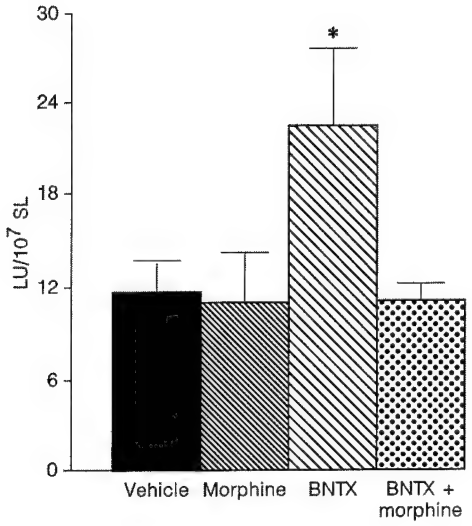

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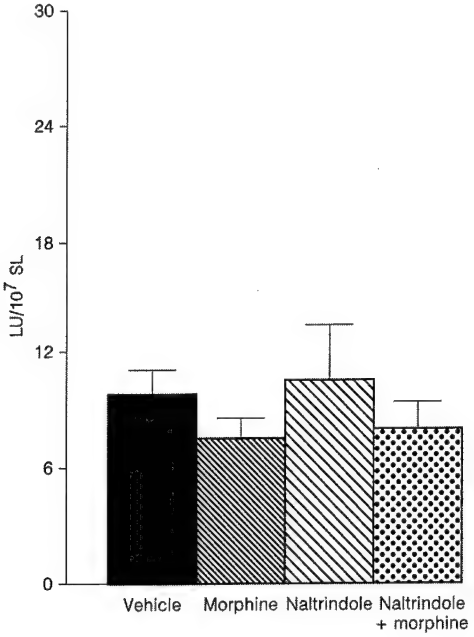

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Fig. 3. BNTX antagonizes morphine-mediated suppression of peritoneal CTL activity. Mice (n = 9/group) were treated as described in the legend to figure 2. PL were assayed for CTL activity using ^{51}Cr-labeled EL-4 cells. * p = 0.06 comparing morphine-treated to vehicle-treated controls. Bars represent SEM.

Fig. 4. Morphine antagonizes BNTX-mediated augmentation of splenic NK activity. Mice (n = 10/group) were treated as described in the legend to figure 2. SL were assayed for NK activity using ^{51}Cr-labeled YAC-1 cells. Chronic BNTX exposure to mice resulted in potentiation of splenic NK activity. When mice were coadministered morphine and BNTX, splenic NK activity maintained levels similar to vehicle- or chronic morphine-treated animals. * $p < 0.05$ comparing morphine-treated to vehicle-treated group as determined by ANOVA and Tukey's post t test. Bars represent SEM.

Fig. 5. Naltrindole has no effect alone or in combination with morphine on splenic NK activity. Mice (n = 6/group) were treated as described. SL were assayed for NK activity using ^{51}Cr-labeled YAC-1 cells. Bars represent SEM.

(fig. 2) or PL (fig. 3) CTL activity. However, BNTX treatment alone resulted in a significant increase in splenic NK activity (fig. 4). In animals chronically treated with both morphine and BNTX, no measurable increase in splenic NK activity was observed (fig. 4).

Naltrindole Does Not Antagonize Morphine-Mediated Suppression of CTL Activity

In order to determine the generality of the effects of BNTX, another δ-selective antagonist, naltrindole was investigated under the same conditions used for BNTX.

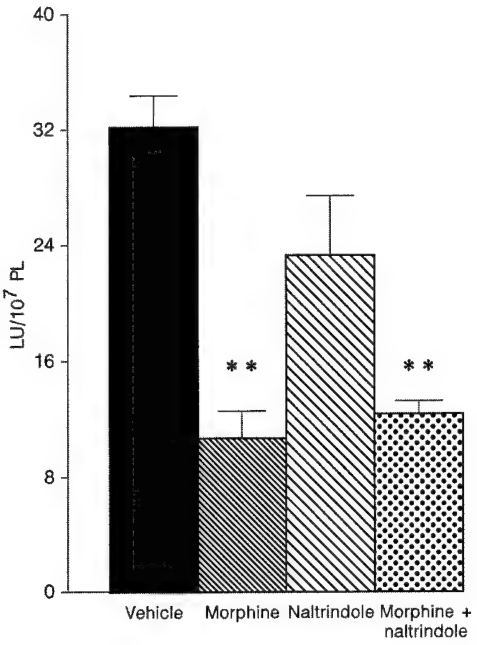

Fig. 6. Naltrindole does not antagonize morphine-mediated suppression of PL CTL activity. Mice (n = 6/group) were treated as described. PL were recovered following the sacrifice of the animals and assayed for CTL activity using ^{51}Cr-labeled EL-4 cells as targets. ** $F(3,23) = 15.4755$, $p < 0.01$ comparing morphine and morphine + naltrindole to vehicle groups as determined by ANOVA and Scheffé's post hoc multiple comparison test. Bars represent SEM.

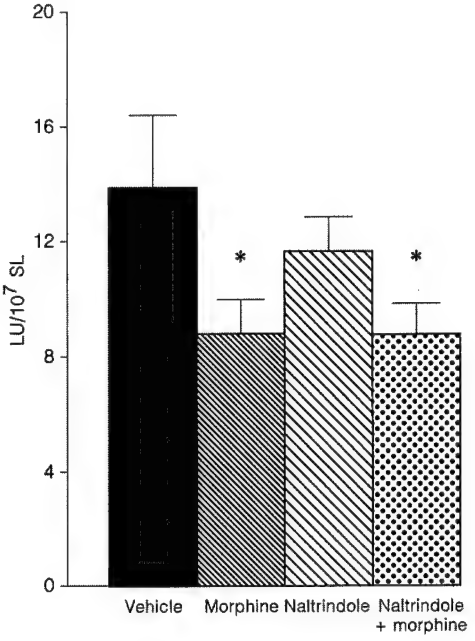

Fig. 7. Naltrindole does not antagonize morphine-mediated suppression of SL CTL activity. Mice (n = 6/group) were treated as described. SL were collected and assayed for CTL activity using ^{51}Cr-labeled EL-4 cells as targets. *$F(3,23) = 2.4238$, $p < 0.05$ comparing morphine and morphine + naltrindole to vehicle groups as determined by ANOVA and Tukey's post hoc t test. Bars represent SEM.

Unlike BNTX, naltrindole had no effect alone on SL NK activity (fig. 5) nor did it antagonize the suppressive effect mediated by morphine on PL (fig. 6) or SL (fig. 7) CTL activity. Consistent with previous observations, chronic morphine exposure was found to significantly reduce the cellularity of the spleen (table 1). Co-administration of naltrindole (20.0 mg/kg, s.c.) to mice chronically exposed to morphine (50.0 mg/kg) partially antagonized this effect (table 1).

Chronic Morphine Treatment Results in Thymic Atrophy but Not a Disproportionate Increase or Decrease in the Percentage of Thymocyte Subpopulations

Since our results indicated morphine exposure suppresses a central T cell function, we investigated the progenitor T cells found in the thymus. Chronic morphine treatment significantly reduced the absolute number of cells recovered from the thymus. Specifically, there was a

Table 1. Naltrindole partially antagonizes morphine-mediated suppression in absolute cell numbers in the spleen[a]

Treatment	Cell number (mean ± SEM)
Vehicle	$545 \pm 0.40 \times 10^7$
Morphine	$2.87 \pm 0.33 \times 10^{7*}$
Naltrindole	$6.01 \pm 0.89 \times 10^7$
Morphine + naltrindole	$4.15 \pm 0.54 \times 10^7$

[a] C3H/HeN mice (n = 6/group) were treated as described. Following sacrifice of the animals, SL were recovered and counted using trypan blue exclusion dye. Less than 2% of the cells were stained with trypan blue.

* $F(3,23) = 8917$, $p < 0.05$ comparing vehicle to morphine group as determined by ANOVA and Scheffé's post hoc multiple comparison test. All other groups were insignificant ($p > 0.05$) relative to vehicle counts.

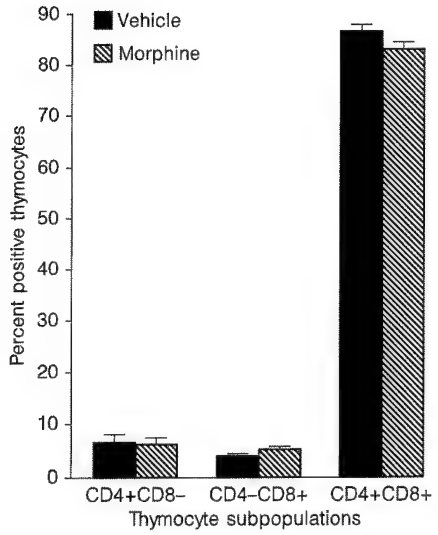

Fig. 8. Chronic morphine exposure does not modify the percentage of CD4+CD8–, CD4–CD8+ or CD4+CD8+ thymic subpopulations. Mice (n = 6/group) were treated as described in the legend to figure 2. Upon sacrifice of the animals, thymocytes were collected, labeled as described and analyzed by FACS. Bars represent SEM.

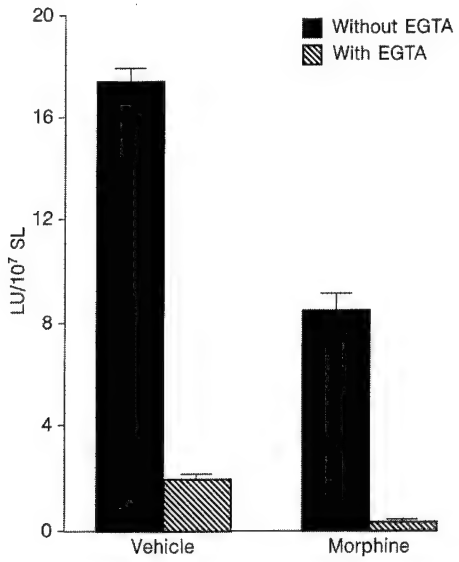

Fig. 9. SL CTL activity is Ca^{2+} dependent. SL obtained from either vehicle- or chronic morphine-treated mice (n = 3/group) were assessed for a Ca^{2+} requirement during the 'lethal hit' by adding 3.6 m*M* EGTA to the media during the 4-hour microcytotoxicity assay. Bars represent SEM.

60% decrease in recovered cells from chronic morphine-treated mice ($5.5 \pm 0.6 \times 10^7$ thymocytes) compared to vehicle-treated controls ($13.38 \pm 0.98 \times 10^7$ cells) ($p < 0.01$, ANOVA and Scheffé). However, the percentage of CD4+CD8–, CD4–CD8+ and CD4+CD8+ cells within the thymus did not change (fig. 8).

CTLs Generated in the Alloimmunized C3H/HeN Mice Lyse Their Targets through a Ca^{2+}-Dependent Mechanism

There are currently two proposed mechanisms of lymphocyte-driven cytotoxicity: (i) nonsecretory and (ii) secretory [16]. CTLs operate through either the Ca^{2+}-dependent membranolytic pathway [20] or a receptor-driven apoptotic-inducing mechanism which does not involve extracellular Ca^{2+} [16]. To identify which mechanism of CTL-directed cytolysis of target cells was utilized by the effector cells, SL were assayed for cytolysis of EL-4 target cells in the presence or absence of EGTA. The results show SL from morphine- and vehicle-treated animals lyse their targets through a Ca^{2+}-dependent process (fig. 9).

Discussion

Consistent with previous findings, the present study shows chronic treatment of mice with morphine resulted in a lower immune response to alloimmunization as reflected by CTL activity in both PL and SL populations. Pretreatment of mice with the δ_1-selective opioid receptor antagonist BNTX [21] did not block morphine-induced suppression of SL CTL activity but did antagonize morphine-mediated suppression of PL CTL activity. However, naltrindole did not antagonize morphine-mediated suppression of SL or PL CTL activity. The data suggest morphine-mediated suppression of SL CTL activity is not mediated through δ opioid receptors. However, the results showing BNTX antagonizes morphine-mediated suppression of PL CTL activity similar to β-funaltrexamine seems to suggest either both δ_1 and μ opioid receptors are involved either centrally (brain) or peripherally (spinal) or BNTX exhibits a peculiar activity not normally associated with δ opioid receptor antagonists. In support of the former notion, naltrindole has recently been shown to antagonize the δ_2-selective opioid agonist [D-Ser^2, Leu^5,

Thr^6] enkephalin- but not the δ_1-selective opioid agonist [D-Pen^2, D-Pen^5]enkephalin-mediated analgesia, even though naltrindole blocks both agonist binding to brain δ opioid receptors suggesting the naltrindole functionally antagonizes δ_2 opioid receptor events [22].

The data indicating that morphine blocks the augmentation in splenic NK acitivity following chronic BNTX administration seems to suggest μ and δ opioid receptors may share a common pathway distal to the opioid binding site. The level of this interaction may be related to the level of action of these compounds. BNTX has been shown to act spinally through δ_1-type opioid receptors [18] which may also include μ_1 opioid receptors, while morphine binds preferentially to μ_2 opioid receptors [23]. Although μ and δ opioid ligands have different sites of action within the central nervous system relative to inducing or antagonizing analgesia, the relationship of the opioid receptor types on immunocompetence in vivo has not been determined. Recently, two studies suggest opioid-mediated analgesia and immunomodulation are functionally independent. Specifically, the administration of morphine into the anterior hypothalamus has been shown to inhibit blood lymphocyte proliferation but has no measurable analgesic action [24]. In a second study, mice pretreated with naltrexone (10.0 mg/kg, s.c.) and subsequently administered increasing increments of morphine up to 100.0 mg/kg showed appreciable analgesia (50–60% of maximal effect) but no suppression of splenic NK activity [17]. Taken together, it is tempting to speculate the existence of opioid receptors which can be distinguished by analgesic versus immunomodulatory activities. This being the case, it may be possible to identify an opioid compound which induces analgesia without the immunosuppressive side effects. Recently, one such compound, OHM3295, a fentanyl derivative, has been found to induce analgesia without suppressing splenic NK activity through a naltrexone-sensitive pathway [25]. In fact. OHM3295 augmented splenic NK activity in a dose-dependent fashion. Consequently, future work is necessary to determine the action of central (supraspinal) and peripheral (spinal) opioid receptors relative to analgesia and immunocompetence.

The present investigation shows that mice administered BNTX daily over 11 days had elevated levels of splenic NK activity. This increase could be due to a redistribution of NK cells from the circulation into the spleen or the activation of pre-NK cells to fully competent cytolytic cells. The observation that mice chronically treated with an opioid antagonist have elevated levels of splenic NK activity is not a novel finding. A previous study has shown mice chronically treated (168 h) with naloxone (0.1–1.0 mg/kg, s.c.) displayed increased levels of splenic NK activity compared to vehicle- or (+)-naloxone-treated control mice [26]. Taken together, the results imply endogenous opioid pathways are important in the regulation of immune homeostasis.

In the present study, SL CTL effector cells incubated with EGTA during the ^{51}Cr-release microcytotoxicity assay did not exhibit cytolytic activity suggesting a requirement for Ca^{2+}. These results complement previous work showing SL from morphine-treated mice possess significantly lower levels of serine esterases [14] which are utilized in the Ca^{2+}-dependent, secretory CTL-directed lysis of target cells [16]. Therefore, the membranolytic pathway used by effector cells generated in the alloimmunized C3H/HeN mice is altered following chronic morphine treatment. Currently, it is not known at what level the modification in the membranolytic pathway is affected by morphine. However, an aberrant response in the generation of cAMP following exposure to alloantigen by CD8+-enriched effector cells taken from morphine-treated animals has been reported [14].

Morphine-mediated suppression of CTL activity is not simply due to a direct interaction of drug with lymphocytes. Although lymphocytes have been shown to possess opioid receptors [27] and recently, an orphan opioid receptor has been cloned and sequences from murine splenic lymphocytes [37], morphine was found to have no effect on the generation of CTLs in one-way MLCs. Therefore, similar to morphine-mediated suppression of splenic NK activity, morphine-induced suppression of CTL activity is indirect, potentially involving the hypothalamic-pituitary adrenal axis [28] and/or the sympathetic nervous system [29, 30].

The occurrence of thymic atrophy following morphine exposure has previously been described by numerous laboratories [28, 31, 32]. In one investigation, the administration of morphine resulted in a time-dependent decrease in the CD4+CD8+ thymocyte population which recovered to normal levels by day 10 [32]. Consistent with these findings, our results show no change in the percentage of total double-positive thymocytes following the sacrifice of mice on day 11. The relationship between the initial decrease in thymocyte population following morphine exposure and the generation of CTLs in the spleen and peritoneum is currently unknown. However, observations showing elevated levels of CD4+ and CD8+ SL in the chronic morphine-treated mice [14] suggest an overcompensation in the peripheral T cell population. This overcompensation might, in part, be due to the inability of the

immune system from the morphine-treated mice to clear the antigen. The clearance defect could be the result of dysfunctional cytolytic activity mediated by the CTL effector cells as reported in the present study, inappropriate processing and presentation of antigen to pre-CTLs by macrophages, or a combination of both processes. Previous studies indicate a decrease in the index of phagocytosis by peritoneal and splenic macrophages following morphine exposure substantiating the notion of a clearance defect [33–35].

To determine the effect of the frequency of exposure to morphine in alloimmunized mice on CTL and NK activity, a recent study has shown a single exposure to morphine (50.0 mg/kg, s.c.) 2 h prior to alloantigen immunization can significantly reduce (40–50%) PL but not SL CTL activity through a naltrexone-sensitive pathway [Carr et al., submitted]. However, a subchronic exposure (daily for 5 days) to morphine (50.0 mg/kg, s.c.) was not found to modify CTL activity in alloimmunized mice suggesting morphine effects on CTL activity are elicited through a complex cascade of events which have not been elucidated.

In summary, the present study indicates the repeated exposure to morphine over an extended period of time (daily for 11 days) has detrimental consequences on CTL activity. It is tempting to speculate that this diminution in effector cell function may in part contribute to the elevated risk among opioid abusers for the acquisition of viral infections. Controlled, experimental studies have shown mice exposed to morphine succumb to viral infections earlier and in greater numbers than vehicle-treated controls [14, 36]. These findings are consistent with published results in the human population [4, 5] supporting the supposition of opioids as cofactors for viral infections including AIDS [6]. Future work is required to identify the intracellular signalling pathways of effector cells modified by opioids following in vivo exposure as well as the neuroendocrine signals elicited by morphine either centrally or peripherally which ultimately affect the immune system. Pending the outcome of these findings, pharmacological intervention seems possible either through the development of novel analgesics which do not activate those neuroendocrine pathways involved in immunomodulation or chemical antagonists which block the opioid-induced immunosuppression but not the analgesia.

Acknowledgments

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Acute Exposure to Morphine Suppresses CTL Activity

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SUMMARY

Chronic exposure (11 days) to morphine has previously been shown to suppress splenic and peritoneal cytotoxic T lymphocyte activity through μ opioid receptors. The present study was undertaken to assess the effects of varying the frequency of exposure to morphine on cytotoxic T lymphocyte activity in C3H/HeN mice immunized with C57BL/6 splenocytes. Mice subchronically treated with morphine (50.0 mg/kg) showed no measurable suppression of splenic natural killer activity or in splenic or peritoneal cytotoxic T lymphocyte activity. However, mice treated acutely with 50.0 mg/kg of morphine exhibited a significant suppression in peritoneal but not splenic cytotoxic T lymphocyte activity. Naltrexone pretreatment of mice receiving an acute dose of morphine blocked the suppression implicating the involvement of opioid receptors. Using column depletion chromatography, peritoneal exudate cells mediating cytotoxic T lymphocyte activity were both $CD4^+CD8^-$ and $CD4^-CD8^+$. Collectively, the results suggest the duration of opioid (morphine) exposure differentially affects peritoneal cytotoxic T lymphocyte activity. These results may have important implications regarding immunity to viral infections in individuals who abuse drugs such as heroin.

INTRODUCTION

The increased incidence in viral hepatitis in parenteral drug users implicates drugs of abuse (narcotics) as co-factors in this disease (Dismukes et al, 1968). Other laboratory studies have demonstrated chronic morphine exposure significantly reduced the survival time of mice infected with encephalomyocarditis virus (Lorenzo et al, 1987), Friend virus (Starec et al, 1991) and herpes simplex virus type I (Carpenter et al, 1994). The decrease in survival following virus infection in mice administered morphine was also found to be correlated with a reduced capacity to secrete interferon-gamma (IFN-γ) (Lorenzo et al, 1987).

Previously, our laboratory has shown the daily administration of morphine (50.0 mg/kg) over 11 days significantly suppressed the generation of cytotoxic T lymphocytes (CTLs) in both the splenic and peritoneal lymphocyte populations in alloimmunized mice without affecting splenic natural killer (NK) activity (Carpenter et al, 1994). Furthermore, the production and secretion of serine esterases by $CD8^+$ CTLs was significantly reduced compared to vehicle-treated controls. The morphine-induced suppression in splenic CTL activity was blocked by pretreatment of mice with the μ-opioid receptor selective antagonist ß-funaltrexamine (Carpenter & Carr, 1995) but not by pretreatment of mice with the δ-opioid selective antagonists naltrindole or (E)-7-benzylidine-7-dihydronaltrexone (Carr & Carpenter, 1995). In the present study, the frequency of exposure to morphine on the NK

and CTL activity in alloimmunized mice was examined. We report that single, but not multiple (daily over 5 days) treatment with 50 mg/kg of morphine significantly suppresses peritoneal but not spleen CTL activity in alloimmunized mice.

MATERIALS & METHODS

Mice and tumor lines

Female C57BL/6 and C3H/HeN mice (Harlan-Sprague Dawley, Indianapolis, IN) 20-22 grams were housed in groups of 6-10 per cage and maintained on a 12 h light/dark cycle. Access to food (Purina Mouse Chow) and water was *ad libitum*. Animals used in these studies were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals Resources, National Research Council, Department of Health, Education, and Welfare Publication Number (National Institutes of Health) 85-23, revised 1985.

The YAC-1 mouse lymphoma line, P815 mastocytoma cell line, and EL-4 lymphoma cell line were obtained from the American Type Culture Collection (ATCC, Rockville, MD); the EL-4 and YAC-1 cells have been maintained in culture by biweekly passage over a 5-6 month period. The P815 mastocytoma has been carried in DBA/2 (Harlan Sprague-Dawley) mice.

Splenic lymphocyte and peritoneal exudate cell preparation

All mice were sacrificed by CO_2 asphyxiation and peritoneal lavage was performed using 10 ml of Hank's balanced salt solution (HBSS). Cells were collected by recovery of peritoneal lavage fluid through a 20 gauge needle and a 10 ml syringe. Spleens were surgically removed and cell suspensions were prepared by

mechanical dispersion. Splenic lymphocytes and peritoneal exudate cells (PEC) were washed with HBSS (250 x g, 5 min) and resuspended in 1 ml of 0.84% NH_4Cl for 2 min. Cells were subsequently washed with 7 ml of HBSS (250 x g, 5 min) and resuspended in RPMI-1640 medium (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (Gibco) and 2.5 % Hybri-max (Sigma Chemical Company, St. Louis, MO) antibiotic/antimycotic solution (complete medium). Cells were counted and examined for viability using trypan blue.

$CD4^+$ and $CD8^+$ enrichment

Mouse T cell subset enrichment columns kits (R & D Systems, Minneapolis, MN) were prepared as suggested by the manufacturer. Peritoneal exudate cells (PEC) from the saline-treated group were pooled as were PEC from the morphine treatment group and separately applied to CD4 and CD8 enrichment columns. Recovered T cells were then assayed for CTL activity using a 4-hr ^{51}Cr release microcytotoxicity assay. Enrichment for $CD4^+$ and $CD8^+$ cells consistently ranged between 70 ± 6 %. $CD4^+$-enriched cells contained less than 2% $CD8^+$ cells and $CD8^+$-enriched cells contained less than 1% $CD4^+$ cells. PECs gated in the mononuclear cell fraction were 6.9 ± 0.2 % $CD4^+$ and 13.6 ± 1.1 $CD8^+$ as determined by flow cytometry (data not shown).

^{51}Cr release cytolytic assay

Splenic lymphocytes were assayed using a 4-hr ^{51}Cr release microcytotoxicity assay as previously described (4). YAC-1 cells were used as targets to measure NK activity in the splenic population while EL-4 (H-2^b) cells were used as targets to measure CTL activity in the splenic lymphocyte and PEC populations. To determine the antigen specificity of the CTLs, ^{51}Cr-labeled P815 mastocytoma cells (H-2^d) were used as third party allogeneic cells in the microcytotoxicity assay. Numbers are expressed in lytic units. One lytic unit is defined as the reciprocal to the number of lymphocytes required to lyse 20% of the targets expressed per 10^7 total spleen cells.

Experimental design

In the first experiment (acute exposure), C3H/HeN mice (n = 12/group) were administered morphine (50.0 mg/kg) or vehicle (HBSS) subcutaneously (s.c.) 2 h prior to receiving 1-2 X 10^7 C57BL/6 spleen cells intraperitoneally (i.p.). Mice were sacrificed 5 days after immunization and PEC and splenic lymphocytes were collected and assayed for CTL (PEC and splenic lymphocytes) and NK (splenic lymphocytes only) activity. The results are the summary of 4 independent experiments using n = 3 mice/group/experiment.

In the second experiment (subchronic exposure), C3H/HeN mice (n = 9/group) were administered vehicle or morphine (50.0 mg/kg) s.c. 2 h prior to receiving 1-2 X 10^7 C57BL/6 spleen cells i.p. Following the immunization, mice received vehicle or

morphine daily for an additional 4 days. On the fifth day, the mice were sacrificed and PEC and splenic lymphocytes were assayed for CTL activity; splenic lymphocytes were also assayed for NK activity. The results are the summary of 3 independent experiments using n = 3 mice/group/experiment.

In the third experiment, C3H/HeN mice (n = 5/group) were administered vehicle or naltrexone (10.0 mg/kg) s.c. 30 min prior to receiving vehicle or morphine (50.0 mg/kg) s.c. Two hrs after morphine administration, the mice received 1-2 X 10^7 C57BL/6 spleen cells i.p. Five days after immunization, the mice were sacrificed and PEC were collected and assayed for CTL activity. The results are the summary of 2 experiment using n = 2-3 mice/group/experiment.

Reagents

Morphine sulfate was provided by the Research Technology Branch of The National Institute on Drug Abuse (Rockville, MD). Naltrexone was obtained from Sigma Chemical Company (St. Louis, MO). All compounds were dissolved in vehicle (Hank's balanced salt solution, HBSS) immediately prior to their use.

Statistics

One-way analysis of variance (randomized, block design) was used together with Scheffe post hoc multiple comparisons test or Tukey's t-test to determine significance ($p < .05$) between the saline and drug-treated groups. The statistical

package used the GBSTAT program (Dynamic Microsystems Inc., Silver Springs, MD).

RESULTS

An acute but not subchronic exposure to morphine suppresses peritoneal lymphocyte CTL activity. Previous studies indicated that chronic exposure (daily over 11 days) to 50.0 mg/kg of morphine significantly suppresses splenic and PEC CTL activity (Carpenter et al, 1994; Carpenter & Carr, 1995). To determine the effect of the duration to morphine exposure on NK and CTL activity in alloimmunized mice, C3H/HeN mice were administered morphine once before immunization (acute) or daily over a 5 day period before and after immunization (subchronic). No discernible effect on CTL cytolytic activity of splenic or PEC populations was noted in the subchronic morphine-treated mice compared to vehicle-treated controls (Table I). Likewise, there were no changes in splenic natural killer (NK) activity comparing the drug- and vehicle-treated groups of animals (Table I). However, in mice given a single dose of 50.0 mg/kg morphine two hours prior to alloimmunization, there was a significant diminution in PEC CTL activity compared to vehicle-treated controls (Table II). No differences were observed when splenic NK and CTL activity between the two groups of mice were compared (Table II). Mice pretreated with 10.0 mg/kg of the opioid receptor antagonist naltrexone exhibited no suppression in PEC CTL activity following acute morphine (50.0 mg/kg, s.c.) treatment (Fig. 1) indicating the drug-induced suppression is mediated through opioid receptors. Naltrexone (10.0 mg/kg) alone had no effect on CTL activity (Fig. 1).

To determine the antigen specificity of the PEC effector population, cells isolated from the peritoneal cavity were assayed for allospecificity using a third-party target. Relative to cytolysis of EL-4 targets, there was modest activity against P815 ^{51}Cr-labeled target cells (Fig. 2).

<u>$CD4^+CD8^-$ and $CD4^-CD8^+$ PEC exhibit CTL activity.</u>

Previous studies using this model system showed that splenic CTL activity is mediated solely by $CD4^-CD8^+$ cells (Carpenter et al, 1994). To identify the effector cells in the PEC population, PECs from vehicle- and morphine-treated mice were enriched for $CD4^+CD8^-$ and $CD4^-CD8^+$ lymphocytes using a column enrichment technique. Both the $CD4^+$-enriched and $CD8^+$-enriched PEC populations exhibited CTL activity as determined by cytolysis of EL-4 cells (Table III). Similar to the unfractionated PEC from morphine-treated mice, $CD8^+$-enriched PEC displayed a depressed CTL activity compared to $CD8^+$-enriched PEC from vehicle-treated mice (Table III). However, there was no significant difference detected in CTL activity in the $CD4^+$-enriched cells from the PEC obtained from morphine-treated compared to vehicle-treated animals (Table III). In addition, there was no discernible increase in lytic units in the enriched populations compared to the unfractionated PEC population suggesting that yet another unidentified population might also be involved in cytolysis of EL-4 targets.

DISCUSSION

In the present study, acute (1 exposure) but not subchronic (daily administration over 5 days) exposure to 50 mg/kg of morphine in alloimmunized C3H/HeN mice significantly suppressed PEC CTL activity through a naltrexone-sensitive pathway. These results suggest that a single exposure to narcotics such as morphine can have a significant effect on the outcome of an antigen-driven immune response. However, the short but continuous exposure to morphine (subchronic treatment) seems to tolerize the immune system as well as the hypothalamic-pituitary adrenal (HPA) axis to the action of the drug. Specifically, the present study shows that subchronic exposure to morphine has no effect on NK or CTL activity while acute morphine administration suppresses CTL activity. Other investigations have yielded similar results following subchronic morphine exposure and measuring phagocytosis (Levier et al, 1993), spleen atrophy and lipopolysaccharide-induced lymphocyte proliferation (Bryant et al, 1988), and antibody production (Bussiere et al, 1993). The HPA axis has been shown to be activated following morphine administration as measured by elevations in serum corticosterone levels as shown other studies (Bryant et al, 1991; Freier & Fuchs, 1994). However, the elevation is short-lived since by 120 hrs after subchronic exposure similar levels in corticosterone are observed between vehicle- and morphine-treated mice. We also found that corticosterone levels were similar between vehicle- and morphine-treated mice 120 hr post morphine exposure (unpublished observation). A

previous study has also shown that elevations in serum corticosterone levels did not contribute to the suppression of mitogen-induced lymphocyte proliferation following acute morphine administration (Bayer et. al., 1990). Taken together, these observations suggest other pathways unrelated to the HPA axis are also involved in morphine-mediated immunosuppression. This notion is substantiated by the recent observations showing that serum corticosterone levels were significantly reduced in chronic (daily administration for 11 days) morphine (50 mg/kg)-treated C3H/HeN mice relative to vehicle-treated controls (Carpenter et al, 1994). In fact, we have shown central α_1 adrenergic pathways are involved in morphine-mediated suppression of NK activity (Carr et al, 1994) in morphine-mediated immunosuppression. Consequently, these pathways either alone or in concert with the HPA axis may modify immunocompetence following chronic morphine exposure. Chronic morphine exposure (defined as continuous or repeated exposure greater than 7 days) has been shown to significantly suppress (i) CTL activity in alloimmunized mice (Carpenter et al, 1994), (ii) hypersensitivity reactions in previously immunized swine (Molitor et al, 1992), and peripheral blood mononuclear cell NK activity in rhesus monkeys (Carr & France, 1993). Although tolerance may be achieved in animals continuously exposed to morphine, cells of the immune system particularly cell-mediated events (Molitor et al, 1992) must be sensitive to long-term opioid exposure as evidenced by these studies .

In the present study, the effector populations mediating CTL activity in the PEC were both $CD4^+CD8^-$ and $CD4^-CD8^+$. However, the enriched populations did not exhibit elevated cytolytic activity as expected and previously observed in the splenic CTL effector cells (Carpenter & Carr, 1995). This observation may indicate that an, as yet, unidentified PEC population which is lost during the enrichment procedure is also involved in mediating antigen-specific cytolysis. It is tempting to speculate that perhaps macrophages (through the release of soluble factors [cytokines]) might be indirectly involved since this cell population would be separated from the $CD4^+$ or $CD8^+$ cells during the enrichment procedure.

The mechanism by which a single exposure to morphine dramatically suppresses PEC CTL activity is presently unknown. Although opioid receptors mediate the suppression of CTL activity based on naltrexone antagonism of the response, the type (δ, κ, or μ) of opioid receptor involved as well as the level of involvement (peripheral versus central/immune versus neural) has not been determined. Since dendritic cells and macrophages are the antigen presenting cells during primary immunization and morphine exposure has previously been shown to suppress phagocytosis by peritoneal macrophages (a mechanism necessary for antigen processing and presentation) (Szabo et al, 1993), it is possible that morphine alters macrophage functions which are involved in the generation of CTLs. However, it has previously been shown that chronic exposure to morphine does not alter the number of splenic CTLs but rather the cytolytic process itself (Carpenter et al,

1994). Whether the absolute number of effector cells is altered in morphine-treated mice or the cytolytic machinery is dysfunctional following morphine exposure was not addressed in this study. However, the data showing $CD8^+$-enriched but not $CD4^+$-enriched effector cell CTL activity is reduced following morphine treatment indicates the selectivity of morphine. The duration of exposure to morphine has a significant impact on CTL activity which appears to be independent of the activation of the HPA axis (unpublished observation) and may relate to the innervation of lymphoid organs including the mesenteric lymph nodes and spleen.

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Table I. Subchronic Morphine Exposure Does Not Effect CTL or NK Activity[a]

Treatment	Spleen NK	Spleen CTL	Peritoneal CTL
Vehicle	9.4 ± 1.2[b]	10.2 ± 1.8	55.5 ± 5.3
Morphine	12.0 ± 2.0	12.9 ± 4.1	55.0 ± 7.8

[a]C3H/HeN female mice (n = 9/group) were given 50.0 mg/kg of morphine (s.c.) or vehicle and two hours later alloimmunized with 1 X 10^7 C57BL/6 splenic lymphocytes. Mice received either vehicle or morphine (50.0 mg/kg) s.c. daily for four additional days. Five days after administration of alloantigen, the mice were sacrificed and splenic and peritoneal exudate cells were collected and assayed for CTL and NK activity using ^{51}Cr-labeled EL-4 and YAC-1 cells as targets respectively. This table is a summary of three independent experiments using n = 3 mice/group/experiment.

[b]Numbers are in lytic units ± SEM.

Table II. Acute Morphine Exposure Suppresses Peritoneal CTL Activity[a]

Treatment	Spleen NK	Spleen CTL	Peritoneal CTL
Vehicle	12.4 ± 1.1[b]	19.4 ± 1.6	44.8 ± 7.9
Morphine	13.4 ± 1.4	15.6 ± 3.6	20.0 ± 6.0*

[a]C3H/HeN female mice (n = 12/group) were given 50.0 mg/kg of morphine two hours prior to receiving 1 X 10^7 C57BL/6 splenic lymphocytes (i.p.). Five days following the administration of alloantigen, the mice were sacrificed and splenic and peritoneal exudate cells were collected and assayed for CTL and NK activity using ^{51}Cr-labeled EL-4 and YAC-1 cells as targets respectively. This table is a summary of four independent experiments using 3 mice/group/experiment.

[b]Numbers are expressed in lytic units ± SEM.

*$F = 6.1595$, $p < .05$ comparing morphine- to vehicle-treated mice as determined by ANOVA and Scheffe´ multiple comparison test.

Table III. $CD4^+$ and $CD8^+$ Peritoneal Cells Possess CTL Activity[a]

Treatment	Unfractionated	CD4-enriched	CD8-enriched
Vehicle	37.6 ± 1.4[b]	29.9 ± 4.2	32.4 ± 1.4
Morphine	26.1 ± 0.8*	24.2 ± 4.4	24.5 ± 1.5*

C3H/HeN female mice (n = 16/group unfractionated; n = 4/group enriched) were administered 50.0 mg/kg of morphine (s.c.) 2 hr prior to receiving 1-2 X 10^7 C57BL/6 splenic lymphocytes (i.p.). Five days following immunization with alloantigen, peritoneal exudate cells were collected and assayed for CTL activity using ^{51}Cr-labeled EL-4 cells or enriched for $CD4^+$ or $CD8^+$ cells prior to the CTL assay. This table is a summary of four independent experiments using n = 4 mice/group/experiment.

[b]Numbers are in lytic units ± SEM.

*F = 50.7492 (unfractionated) or 14.8242 ($CD8^+$-enriched), $p < .05$ comparing morphine- to vehicle-treated mice as determined by ANOVA and Scheffe´ multiple comparison test.

FIGURE LEGENDS

Figure 1. Naltrexone pretreatment blocks morphine-mediated suppression of peritoneal exudate cell (PEC) CTL activity. C3H/HeN female mice (n = 5/group) were administered 10.0 mg/kg naltrexone (NALT) or vehicle (VEH) s.c. 30 minutes prior to receiving vehicle or 50.0 mg/kg morphine (M) s.c. Two hours after morphine exposure, the mice were immunized with 1-2 X 10^7 C57BL/6 splenic lymphocytes (i.p.). Five days following alloimmunization, the mice were sacrificed and PEC were collected and assayed for CTL activity using ^{51}Cr-labeled EL-4 cells. This figure is a summary of 2 independent experiments using n = 2-3 mice/group/experiment. Bars represent SEM. $^{*}F = 3.7376$, $p < .05$ comparing morphine-treated to all other groups as determined by ANOVA and Tukey's post hoc test.

Figure 2. Peritoneal exudate cells (PEC) are antigen specific. C3H/HeN female mice (n = 5/group) were immunized with 1-2 X 10^7 C57BL/6 splenic lymphocytes (i.p.). Five days following alloimmunization, the mice were sacrificed and assayed for cytolytic activity against ^{51}Cr-labeled EL-4 (H-2^b) or P815 (H-2^d) cells as targets. This figure is a summary of two independent experiments using n = 2-3 mice/group/experiment. Bars represent SEM. $^{**}F = 206.1869$, $p < .001$ comparing lytic units of EL-4 to P815 cytolysis as determined by ANOVA and Scheffe´ multiple comparison test.

18

CELLULAR MECHANISMS INVOLVED IN MORPHINE-MEDIATED SUPPRESSION OF CTL ACTIVITY[1]

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INTRODUCTION

Cellular immunity including MHC-unrestricted natural killer (NK) cells and antigen-specific, class I MHC-restricted cytotoxic T lymphocytes (CTLs) plays a central role in monitoring viral infections and tumor growth (1). NK activity has been shown to be modified by opioid compounds both *in vitro* and *in vivo*. The addition of endogenous opioid peptides (e.g., β-endorphin or [met]-enkephalin) to 4-hr ^{51}Cr-release NK microcytotoxicity assays has been shown to augment NK activity; this augmentation is naloxone-sensitive (2). However, the acute administration of opioid drugs (e.g., morphine or fentanyl) in mice has been shown to suppress splenic NK activity through a naltrexone-sensitive mechanism (3-5). This suppression involves opioid receptors located in the periaqueductal gray matter of the mesencephalon (6). Pretreatment of the mice with the α-adrenoceptor antagonists phentolamine or prazocin blocks morphine-mediated suppression of splenic NK activity implicating α-adrenergic receptor involvement (7). Preadministration of mice with phentolamine (general α-adrenoceptor antagonist) but not doxazosin (peripheral-acting α-adrenergic receptor antagonist) inhibits morphine-mediated suppression of splenic NK activity further implicating central (brain) rather than peripheral α-adrenergic involvement (8). Alternatively, other neuroendocrine hormones may be utilized distal to the brain ultimately influencing NK effector cells. Specifically, splenic serotonin levels are elevated following acute morphine administration and such increases can be blocked by pretreating animals with phentolamine (8). These results suggest serotonin might be solicited by adrenergic processes ultimately resulting in suppression of splenic NK activity. Consistent with this notion, a recent study revealed serotonin suppressed NK activity in whole blood and such effects could be reversed with interferon-α (9). Certainly, this is a complicated issue involving many mediators which may have direct or indirect effects on the NK cells.

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At the cellular level, NK effector cells from acute morphine-treated mice have a reduced capacity to form conjugates with target cells (8). In addition, of those cells which can form conjugates with their targets, a reduced number initiate lysis of targets relative to NK-enriched effector cells from vehicle-treated mice (8). These results are consistent with the observation showing the reduction in splenic NK activity is not due to a redistribution of NK effector cells ($NK1.1^+2B4^+$) from the spleen (8). Taken together, the data suggest acute morphine administration modifies cellular machinery involved in the "lethal hit" of the NK effector cells with their targets. Currently, tyrosine kinase activity is under investigation since previous studies indicate a correlation between tyrosine kinase activity and NK-mediated cytolysis of target cells (10,11).

Contrary to the effects of opioids on *in vitro* and *in vivo* NK activity, less information is available concerning the action of opioids on CTL activity. The generation of CTLs in *in vitro* one-way mixed lymphocyte cultures (MLCs) has previously been shown to be augmented by β-endorphin and [met]-enkephalin in a naloxone-reversible manner (12). However, the *in vivo* effect of opioids on CTL generation or activity is not known. Consequently, a study was undertaken to assess the effect of chronic morphine exposure on CTL activity in alloimmunized mice.

CHRONIC MORPHINE EXPOSURE SUPPRESSES CTL ACTIVITY IN ALLOIMMUNIZED MICE

A study was initiated to investigate chronic (11 days) morphine exposure on NK and CTL activity in alloimmunized mice. Specifically, C3H/HeN ($H\text{-}2^k$ haplotype) mice were administered with morphine (50 mg/kg, s.c.) two hours prior to receiving 1×10^7 C57BL/6 ($H\text{-}2^b$) spleen cells, i.p.. Following the immunization, mice received vehicle or morphine (50 mg/kg) daily for an additional 6 days. On day 7, mice were reimmunized with 1×10^7 C57BL/6 spleen cells, i.p two hours after the administration of vehicle or morphine. Following the second immunization, mice received morphine or vehicle daily for an additional three days. On day 11, the mice were sacrificed and peritoneal exudate leukocytes (PL) and splenic lymphocytes (SL) were assessed for NK and CTL activity. It is our opinion that this treatment regimen more closely reflects the habits of an opioid abuser who must continually administer drug in order to avoid the effects of withdrawal. Chronic exposure to morphine significantly suppressed SL and PL CTL activity as determined using ^{51}Cr-labeled EL-4 cells ($H\text{-}2^b$) in a 4-hour microcytotoxicity assay (13). The SL from either vehicle- or morphine-treated groups showed no measurable cytolysis against third party targets (P815, $H\text{-}2^d$) indicating the antigen-specificity of the target cells. Similarly, SL from non-immunized mice showed no measurable cytolytic activity against ^{51}Cr-labeled EL-4 cells. CD4- and CD8-enrichment studies showed the CTL effector cells were phenotypically-defined as $CD4^-CD8^+$ (33).

Opioid receptor antagonists were used to define the opioid specificity of morphine-mediated suppression of SL and PL CTL activity. Previous studies utilized β-funaltrexamine (β-FNA, μ-selective opioid receptor antagonist), naltrindole (δ-selective opioid receptor antagonist), norbinaltorphimine (κ-selective opioid receptor antagonist), and naloxonazine (μ_1-selective opioid receptor antagonist) to identify the involvement of μ_2-opioid receptors in the suppression of splenic NK activity following acute morphine administration (7). In the present investigation, β-FNA and (E)-7-benzylidine-7-dihydronaltrexone (BNTX, δ-selective opioid receptor antagonist) (14) were used to determine μ versus δ opioid receptor involvement. The pretreatment of mice with β-FNA (40 mg/kg every 72 hours and 18-24 hours prior to receiving morphine) effectively antagonized the suppression of PL and SL

CTL activity elicited by chronic morphine treatment (33). The pretreatment of mice with BNTX did not block morphine-mediated suppression of SL CTL activity (34). However, the mice pretreated with BNTX antagonized morphine-mediated suppression of PL CTL activity (34). Taken together, the results indicate morphine-mediated suppression of SL CTL activity utilizes μ opioid receptors while the type(s) of opioid receptors associated with morphine-mediated suppression of PL CTL activity has not been resolved.

The possibility that morphine might have a direct effect on CTL generation or activity was investigated *in vitro*. Specifically, morphine (10^{-5} - 10^{-11} M) added either during the initiation of culture or daily throughout the one-way MLC showed no effect on the generation of CTLs suggesting morphine does not interact directly with lymphocytes (presumably through opioid receptors) in modifying CTL activity (34).

To define the biological significance of morphine-mediated suppression of CTL activity, mice were infected with the LD_{50} of the McKrae strain of herpes simplex virus in the hind footpad and subsequently administered vehicle or morphine (50 mg/kg) daily over a 21 day period. The results showed chronic morphine-treated animals died earlier and in greater numbers compared with the vehicle-treated controls (13). Consequently, the assertion that morphine might be a co-factor in the acquisition of viral infections including AIDS (15) is supported by this data.

To identify the cellular mechanisms linked to the recognition and lysis of the target cell which might be altered following morphine exposure *in vivo*, a study was undertaken to assess target recognition and selective pathways associated with some of the lytic processes functional during the "lethal hit" of the target by effector cells from vehicle- and chronic morphine-treated mice.

CHRONIC MORPHINE TREATMENT SUPPRESSES GRANULATION AND cAMP RESPONSES TO ALLOANTIGEN BY CTL EFFECTOR CELLS

The recognition of targets by effector cells and subsequent activation of effector cells prior to lysis of targets involves a complex intracellular signalling linguistics (Fig. 1). Initially, the $CD8^+$ effector cell recognizes the peptide antigen association with class I MHC expressed on the surface of the target cell (in this case, the EL-4 [$H\text{-}2^b$] lymphoma) by the T cell receptor (TCR). In addition, the CD8 molecule on the effector cell interacts with the class I molecule of the target increasing the stability of the TCR-MHC class I interaction. Furthermore, the LFA-1 (CD11a) antigen on the effector cell interacts with the integrin counterpart (CD54) of the target further increasing the stability of the effector/target interaction. The interaction of CD11a with CD54 may result in the phosphorylation (serine residue of the β-chain, CD18) of the cytoplasmic domain of CD11a although the result of this phosphorylation has not been conclusively determined. Another surface antigen on the effector cells CD45 may play an extremely important role in cell activation. The phospho-tyrosine phosphatase activity associated with the CD45 molecule may act to dephosphorylate the kinase domain of $p56^{lck}$ and $p53^{fyn}$ src-protein tyrosine kinases (16). Since the $p56^{lck}$ has a amino-terminal cysteine motif which can interact with the CD8 cytoplasmic tail, it places this tyrosine kinase in close proximity to the TCR-CD3 complex. Consequently, the SH2 domain of the $p56^{lck}$ or $p53^{fyn}$ can associate with the phosphorylated Γ or zeta chains of the CD3 complex. A syk-related tyrosine kinase ZAP-70 driven by the phosphorylation of the zeta chain of the CD3 molecule may be involved in activation of phospholipase $C_{\Gamma 1}$ ultimately resulting in the hydrolysis of phosphatidylinositol 4,5 bisphosphate generating inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG). DAG has been shown to

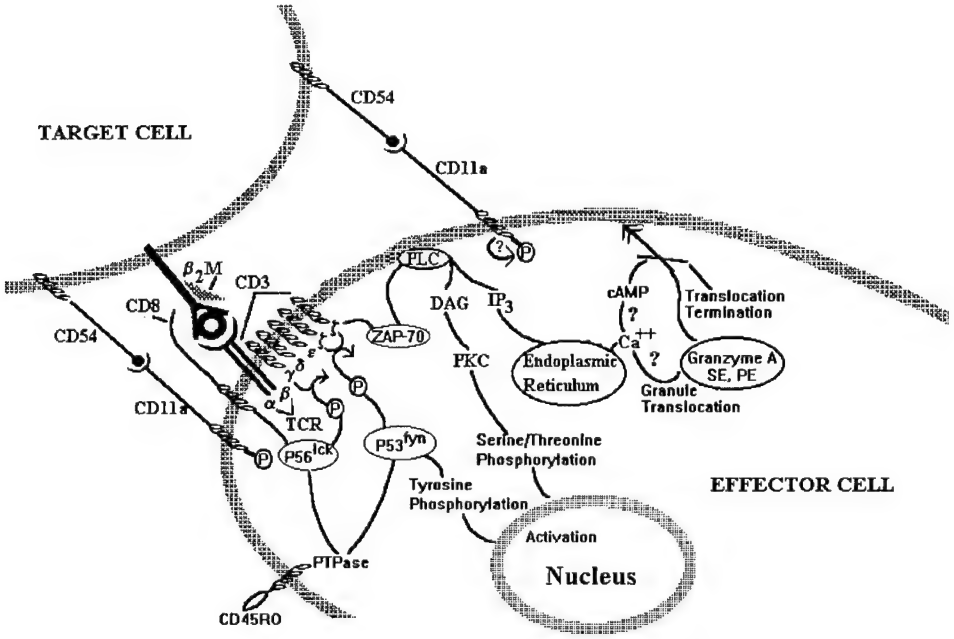

Figure 1. Signalling transduction pathways associated with $CD8^+$ activation following target cell recognition.

transiently stimulate protein kinase C (PKC) resulting in serine/threonine phosphorylation leading to the activation of the effector cell. In addition, IP_3 generation drives the mobilization of Ca^{2+} which through either cAMP-dependent or independent mechanisms increases the translocation of granules to the membrane for delivery to the target. Following the initial recognition of the target, one or more of the intracellular signalling pathways described above could be altered in effector cells from the morphine-treated mice. Previous work has described the suppression of CD2 expression (17) and Ca^{2+} mobilization (18) in the presence of morphine. Since CD2 has been implicated in the regulation of CD45R (16) and Ca^{2+} mobilization is linked to granzyme A-mediated cytolysis of targets by effectors (19), we have explored some of these pathways for possible modification following morphine exposure.

There are currently two proposed mechanisms of lymphocyte-driven cytotoxicity: (i) non-secretory, a receptor-mediated triggering of apoptosis and (ii) secretory, a membranolytic mechanism (19). CTL-mediated cytotoxicity includes both mechanisms, a Ca^{2+}-dependent membranolytic pathway (20) and an apoptotic receptor-driven pathway which may not involve extracellular Ca^{2+} (19). To determine which mechanism is operational in our system, splenic CTLs generated in the above treatment regimen were assessed for cytolysis of targets (EL-4) in the presence of the Ca^{2+}-chelating agent, ethyleneglycol-bis-(β-aminoethylether)N,N'-tetra-acetic acid (EGTA). Splenic CTLs from either chronic morphine- or vehicle-treated mice did not elicit the "lethal hit" in the presence of EGTA as measured by ^{51}Cr release (34) indicating a Ca^{2+}-dependent cytolytic process. The Ca^{2+}-dependent, secretory membranolytic mechanism of target cell lysis involves the release of granules, granzymes, and perforin which function to initiate pore formation in the target cell membrane ultimately resulting in internal disintegration and DNA fragmentation (19). Constituents in granzymes include serine esterases which have been implicated in the cytolytic process of CTLs and NK cells (19). Accordingly, we compared the levels of serine esterases in response to alloantigen from SL of vehicle- and chronic morphine-treated mice. The results show the

percent of serine esterase release was lower in the SL taken from chronic morphine-treated animals compared to serine esterase release from vehicle-treated controls (13). In addition, there was a significant decrease in the total serine esterase content in SL obtained from the chronic morphine-treated mice. These results suggest one means of morphine-mediated suppression of CTL activity is by decreasing the levels and release of cytolytic-associated enzymes by effector cells. Another intracellular event implicated in the degranulation of effector cells is the rise in cAMP (21). In addition, cAMP has been associated with detachment from the target cell as well as a signal responsible for the initiation of recycling by CTLs for subsequent lytic function (21). Consequently, we investigated cAMP levels by $CD8^+$-enriched effector cells following alloantigen exposure *in vitro*. The results show $CD8^+$-enriched effector cells from the chronic morphine-treated mice do not respond with an elevation in cAMP levels following alloantigen exposure compared to cells from vehicle-treated mice. Taken together, the data suggest CTLs from chronic morphine-treated mice have an aberrant intracellular signalling transduction pathway which results in a decrease in serine esterase release ultimately resulting in a reduction in CTL activity.

Previous results show NK-enriched effector cells taken from acute morphine-treated mice conjugate less effectively with their targets compared to NK effector cells from vehicle-treated controls (8). Therefore, we investigated CTL effector cells from chronic morphine- and vehicle-treated groups for their ability to conjugate with targets. The results show CTLs from either morphine- or vehicle-treated mice conjugate equally well with their targets (13). In addition, the expression of CD11a on $CD8^+$ effector cells from vehicle- and morphine-treated mice was at similar levels which correlates with the conjugate studies. Collectively, the data seem to indicate the number of CTLs generated over the 11 day treatment regimen with either vehicle or morphine does not differ. Instead, the suppression in measurable CTL activity is due to a defect in the effector cell-associated lytic mechanism(s).

CHRONIC MORPHINE TREATMENT ALTERS HYPOTHALAMIC PITUITARY ADRENAL AXIS HORMONE LEVELS

Previous studies have shown hypothalamic pituitary adrenal (HPA) axis involvement in immunomodulation following short term exposure to morphine (22-24). Specifically, the administration of morphine elicits a stress response with a subsequent increase in serum corticosterone levels. Based on these results, a study was conducted to measure corticosterone levels in the serum of vehicle- and chronic morphine-treated mice. Interestingly, the results showed serum corticosterone levels were lower in the chronic morphine-treated mice relative to vehicle-treated controls (13). However, these measurements were taken at the end of the treatment regimen on day 11 following the sacrifice of the animals and do not necessarily indicate the potential relationship between the hormone and morphine-mediated suppression of CTL activity. It is tempting to speculate that these levels reflect the tolerance of the mice to morphine since the characteristic straub tail associated with morphine administration was less apparent towards the end of the treatment regimen (days 9 and 10, unpublished observation).

Still another possibility of morphine-mediated immunosuppression might reside in the lymphocyte-derived, proopiomelanocortin (POMC) peptide hormone levels. Specifically, the POMC-derived hormone, β-endorphin has previously been shown to augment CTL activity (12). We hypothesized that chronic exposure to morphine may reduce the level of expression of the POMC transcript encoding β-endorphin and hence, reduce β-endorphin levels. By using reverse transcription-polymerase chain reaction and oligonucleotide prim-

ers selective for exon 3 of POMC (encodes β-endorphin), the results showed no differences in the expression of POMC by SL obtained from either treated group of animals (13). Specifically, SL from 3 of 13 vehicle-treated mice were positive for exon 3 POMC mRNA while the lymphocytes from 6 of 14 chronic morphine-treated mice were also positive. Consequently, the levels of POMC do not correlate with morphine-mediated immunosuppression and probably play no role in this model system.

SUMMARY

Based on a plethora of data from many laboratories, we have proposed the following mechanisms by which morphine alters immune homeostasis and immunocompetence *in vivo* (Fig. 2). Specifically, the administration of morphine subcutaneously via routing through blood interacts directly with opioid receptors on cells of the immune system or on receptors within the central nervous system. Although there is currently no evidence to support the direct involvement of morphine on lymphocyte opioid receptors, *in vitro* studies show the existence of functional, naloxone-sensitive opioid receptors (25). In addition, pharmacological and biochemical characterization of lymphocyte opioid receptors has been shown to be consistent in many instances, with the profile of neural-derived opioid receptors (25-27). Finally, recent molecular studies using oligonucleotide primers specific for the δ-class opioid receptor cloned from NG-108-15 cells (28) have been used in reverse transcription-polymerase chain reactions to generate a 400 bp product in SL which has 100% sequence homology with a published opioid receptor cloned from a brain library (35). However, future studies are necessary to establish the role of lymphocyte opioid receptors following the *in vivo* administration of opioids (e.g. fentanyl, methadone, and morphine).

Since the administration of morphine subcutaneously appears to predominately interact with brain opioid receptors (3) located in the mesencephalon (5), other neuroendocrine systems become candidates for activation and subsequent direct modulation of immune function: (i) the HPA axis and (ii) the sympathetic nervous system (SNS). The activation of the HPA axis through the release of corticotropin releasing hormone elicits the production of adrenocorticotropin hormone (ACTH) by corticotrophs of the anterior pituitary which in turn travels through the blood to the adrenals and elicits the production of corticotropin. Corticotropin can then act on lymphocytes resulting in suppression of selective immune parameters predominately T cell-mediated (29). The activation of the SNS by morphine does modify immune responsivity through the "hard-wiring" of immune organs (thymus, spleen, bone marrow, and lymph nodes) (30). Specifically, studies have shown SNS involvement in morphine-mediated suppression of lymphocyte proliferation as well as interleukin (IL)-2, IL-4, and interferon-Γ production (31), and natural killer activity (7). Whereas central α-adrenoceptors are involved in morphine-mediated suppression of splenic NK activity (8), peripheral β-adrenoceptors are involved in morphine-mediated suppression of lymphocyte proliferation and cytokine production (32). Finally, the consideration of cytokines including neuroendocrine peptide hormones produced by immune cells in response to neural stimulation in the lymphoid organ following morphine administration must also be taken into account. Whether these products or blood borne neuroendocrine hormones including catecholamines and corticosterone ultimately are responsible in the suppression of immunocompetence following morphine administration remains a question. Based on the studies presented herein, we have concluded the SNS and HPA are the predominate sources involved in morphine-mediated effects on the immune system.

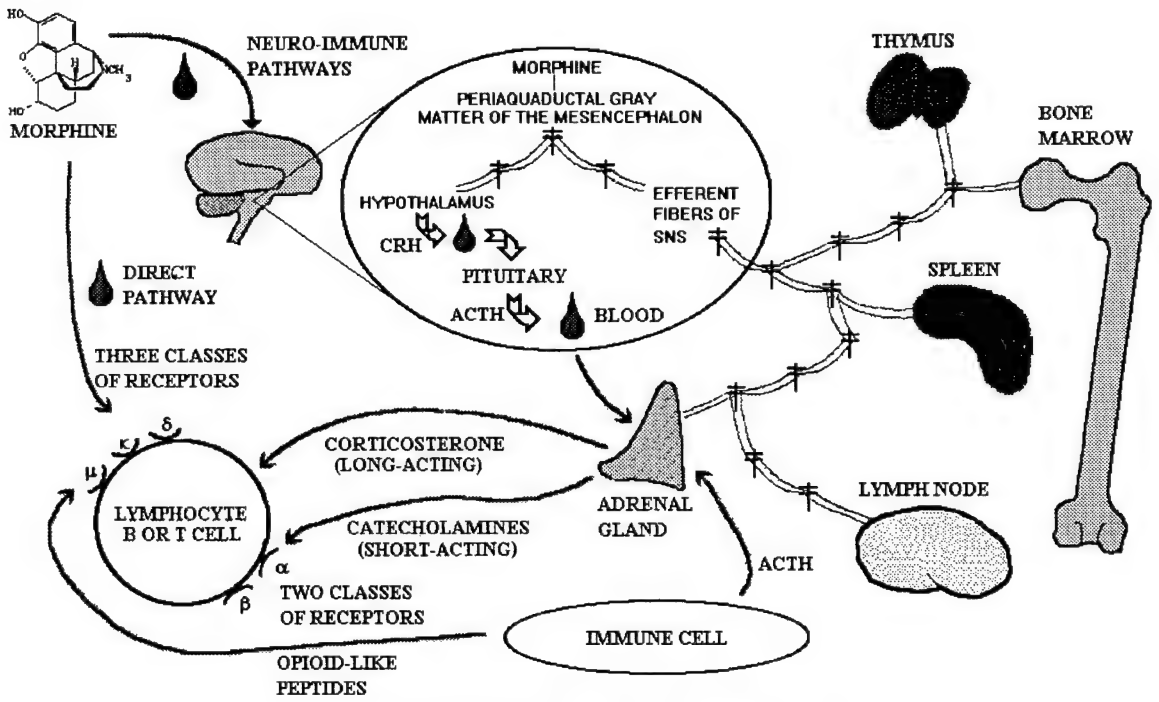

Figure 2. Immunomodulation via opioids. Proposed mechanisms.

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Functional role and sequence analysis of a lymphocyte orphan opioid receptor ☆

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Functional role and sequence analysis of a lymphocyte orphan opioid receptor ☆

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Abstract

Pharmacological evidence indicates that lymphocytes express opioid receptors, but this finding has been questioned. By DNA sequencing of reverse transcription-polymerase chain reaction products, we have found that mouse lymphocytes express mRNA encoding an orphan opioid receptor. These mRNA transcripts were detected in the $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ lymphocyte subpopulations. Northern blot analysis confirmed that splenic lymphocytes express a 1.5-kb orphan opioid receptor mRNA. Fifteen bases encoding Tyr^{71}-Arg^{75} in the first intracellular loop are alternatively spliced, suggesting that orphan opioid receptor mRNA encodes two receptor subtypes. Treatment of lipopolysaccharide-stimulated lymphocytes with orphan opioid receptor antisense oligonucleotides suppressed polyclonal IgG and IgM production by 50%. Our results provide direct evidence that lymphocytes express an opioid-like receptor gene, and suggest that this receptor plays a functional role in immunocompetence.

Keywords: Lymphocyte; Reverse transcription-polymerase chain reaction; Orphan opioid receptor; Antisense oligonucleotide; Alternative splicing

1. Introduction

Opioids have been shown to modify immune responses in vitro and in vivo. In vitro, opioid peptides suppress antibody production (Johnson et al., 1982; Heijnen et al., 1986; Taub et al., 1991), augment natural killer (NK) cell activity (Mathews et al., 1983), increase interferon (IFN)-γ production (Mandler et al., 1986), enhance monocyte-granulocyte chemotaxis (Van Epps et al., 1984), and promote CTL generation (Carr and Klimpel, 1986). These events are blocked by opioid receptor (OR) antagonists, implying that the immunoregulatory effects are mediated through OR–ligand interaction.

The characterization of ORs on cells of the immune system has been problematic. Although early studies suggested the existence of opioid binding sites on leukocytes (Mehrishi and Mills, 1983; Falke et al., 1985), these studies were met with scepticism because unconventional experimental procedures were used and the data was not thoroughly analyzed (Sibinga and Goldstein, 1988). However, more recent studies have shown μ-like and κ opioid binding sites on lymphocytes and macrophages. These binding sites exhibit stereoselectivity, saturability, nanomolar affinity, and ligand-class preference (Madden et al., 1987; Carr et al., 1989; Ovadia et al., 1989; Bidlack et al., 1992). Photoaffinity-labeling techniques have identified δ- (Carr et al., 1988), κ- (Carr et al., 1989), and μ-like (Radulescu et al., 1991) opioid binding sites on leukocytes that structurally resemble their neuroendocrine counterparts (Carr, 1991). Moreover, in one study the purification of an opioid binding moiety that showed ligand selectivity was demonstrated (Carr et al., 1990a). However, the biological relevance of these binding sites remains in question.

☆ This work does not necessarily reflect the position or the policy of the government, and no official endorsement should be inferred.

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The administration of opioids in vivo (e.g. morphine, the prototypic μ ligand) has been shown to alter NK cell activity by indirect routes including the activation of the periaqueductal gray matter of the mesencephalon (Weber and Pert, 1989) and α-adrenergic pathways (Carr et al., 1993; Carr et al., 1994). Other studies have shown morphine exposure results in thymic atrophy (Sei et al., 1991) and decreases in interleukin (IL)-2/IL-4 and IFN-γ production (Lysle et al., 1993). These effects are mediated by the hypothalamic-pituitary adrenal axis and peripheral β-adrenergic action. Taken together, the studies indicated the need to re-evaluate the existence and functional significance of ORs on cells of the immune system.

The development of molecular biological techniques has allowed investigators to clone a variety of neuropeptide receptors including brain δ-, κ-, and μ- ORs (Evans et al., 1992; Kieffer et al., 1992; Wang et al., 1993; Yasuda et al., 1993). Cloning of these ORs has led to the discovery of a new OR class, referred to herein as the orphan opioid receptor (oOR), which shares 66% nucleotide sequence homology with δOR. oOR has been cloned from human (Mollereau et al., 1994), rat (Bunzow and Grandy, 1994; Chen et al., 1994; Fukuda et al., 1994), and mouse (Yasuda et al., 1994) brain cDNA libraries, but these studies have only begun to address the function of oOR.

Consistent with their role as G-protein coupled receptors, ORs possess seven transmembrane (TM) spanning regions whose amino acid sequences are highly conserved amongst δ-, κ-, and μ- ORs (Reisine and Bell, 1993). Based on this homology, oligonucleotide primers were prepared to the TM-encoding sequences of DOR-1, a δOR clone (Evans et al., 1992), and reverse transcription polymerase chain reaction (RT-PCR) was used to screen splenic lymphocyte RNA for OR-encoding transcripts. However, these primers did not amplify δOR mRNA, but rather, amplified the homologous portion of a mRNA that encodes oOR. Our results demonstrate that lymphocytes express oOR mRNA.

2. Materials and methods

2.1. Animals and cells

BALB/c, DBA/2, and ICR-Swiss mice (Harlan-Sprague Dawley, Indianapolis, IN) were used in these experiments. EL-4 mouse thymoma and L929 mouse fibroblast cell lines were obtained from the American Type Culture Collection (Rockville, MD). Lymphocyte suspensions were teased out of the spleen and drawn through a 23-gauge needle to mechanically disperse cells. RBCs were separated from lymphocytes either with 0.84% NH_4Cl, or by gradient centrifugation over Ficoll-Hypaque (Lympholyte M®, Accurate Scientific and Chemical Co., Westbury, NY). Concanavalin A (ConA, 10 μg/ml) stimulation of splenic lymphocytes was performed in RPMI 1640, 10% fetal bovine serum for 48 h at 37° C/5% CO_2 at a density of 5×10^6 cells per ml.

2.2. Isolation of RNA

RNA extraction was performed with Ultraspec® RNA isolation reagent (Biotecx Inc., Houston, TX) according to the manufacturer's instructions. RNA was isolated from mouse brain and whole spleen by homogenization of the entire organ in Ultraspec®. RBC lysis was performed prior to the isolation of poly(A)$^+$ RNA from splenic lymphocytes. For RT-PCR and total RNA blots, lymphocytes were purified with Lympholyte M® prior to RNA extraction. Poly(A)$^+$ RNA was purified from total RNA using a Poly ATtract® mRNA isolation kit (Promega Corp., Madison, WI).

2.3. Cell sorting by FACS

Spleen cells from ICR mice were collected by mechanical dispersion and RBCs were lysed with 0.84% NH_4Cl. The cells were washed with RDF buffer (R & D Systems, Minneapolis, MN) and labeled with rat anti-mouse CD4 (clone RM4-5, Pharmigen, San Diego, CA) conjugated with FITC and rat anti-mouse CD8 (clone 53-6.7, Pharmigen) conjugated with phycoerythrin. The cells were incubated on ice for 30 min and subsequently washed twice with RDF buffer. The labeled cells were then sorted using a Coulter Elite FACS (Coulter, Hialeah, FL). $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ cells (5×10^6 cells) were collected for RT-PCR analysis. $CD4^+$ and $CD8^+$ populations were 98% pure as assessed by FACS.

2.4. RT-PCR and DNA sequencing

First strand cDNA was synthesized from 500 ng total RNA using an oligo-dT_{15} primer and AMV reverse transcriptase (Promega Corp.) according to the manufacturer's instructions. The cDNA template was combined with buffer, 0.25 μM each PCR primer, 100 μM each dNTP, and 2.5 U *Taq* polymerase (Promega Corp.) in a 50-μl reaction volume and overlaid with mineral oil. PCR was accomplished in a MJ Research thermal cycler (Watertown, MA) with 35 cycles of 94° C (1′15″) → 57° C (1′15″) → 72° C (35″). Oligonucleotide PCR primers were synthesized by LSU Medical Center Core Laboratories (LSUMC, New Orleans, LA) and are listed in Table 1. PCR amplification with glyceraldehyde phosphate dehydrogenase primers (G3PDH, Iwai et al., 1991) was performed on an aliquot of each

Table 1
Delta and orphan opioid receptor PCR primers

PCR Primer	Oligonucleotide sequence
δOR395$^+$ (532)	5′ GCT GTG CTC TCC ATT GAC TAC TAC AAC ATG
δOR695$^-$ (832)	5′ CGA AGG CAA AGA GGA ACA CGC AGA T
oOR52$^+$	5′ TCA TTG TGC TCC TGC CTG CCT TTC T
oOR114$^+$	5′ GAG GTT GTG TGT GCT GTT GGA GGA A
oOR925$^-$	5′ GGT CCT TCT CTC GGG AGC CTG AAA G
oOR1292$^-$	5′ ATG GGC AGG TCC ACG CCT AGT CAT G
oOR1333$^-$	5′ CCG TGT TGG GTG TAG ATG GGC TCT G

The number given in each primer refers to the position of the 5′ base. Numbers given in parentheses for δOR primers denote corresponding position of the 5′ base in the oOR sequence. The +/− symbol indicates sense/antisense polarity of primers relative to mRNA.

cDNA sample to ensure that reverse transcription had occurred. PCR products were analyzed on TBE (0.09 M Tris-borate, 1 mM EDTA, pH 8.0), 2% agarose gels. For restriction analysis with *Bsu*36I, oOR114/oOR925$^-$ PCR products were purified with PCR Magic Miniprep columns® (Promega Corp.) and eluted in water. PCR products were digested for 2 h at 37° C with 28 U of *Bsu*36I (Promega Corp.), and electrophoresed on a 2% agarose gel.

Isolation of larger PCR products was done by nested PCR to minimize amplification of extraneous products. Nested PCR was performed by first amplifying for 15 cycles of 94° C (1′15″) → 57° C (1′15″) → 72° C (1′45″) with 2 ng of each outer primer (i.e. oOR52$^+$ and oOR1333$^-$), and then adding 100 ng of each inner primer (i.e. oOR114$^+$ and oOR1292$^-$) and amplifying for another 30 cycles of 94° C (1′15″) → 57° C (1′15″) → 72° C (1′15″).

oOR PCR products were ligated into TA cloning vectors (Invitrogen Corp., San Diego, CA). Transformed *Escherichia coli* were selected on Luria-Bertani carbenicillin (50 μg/ml) agar plates, coated with X-Gal to allow blue/white selection of recombinant clones. Using oOR primers, PCR was performed on an inoculum of each bacterial colony to identify those bearing oOR plasmid clones. Plasmid DNA was obtained from recombinant clones by the alkaline lysis method (Sambrook et al., 1982) and used directly as a template for sequencing. DNA sequencing was done by the Sanger method (Sanger et al., 1977) with a CircumVent sequencing kit (New England Biolabs Inc., Beverly, MA). Sequencing reaction products were labeled by incorporation of [α-^{35}S]dATP (1000 Ci/mmol; Amersham, Arlington Heights, IL). Identification of DNA sequences and comparison to other ORs was achieved by submission of query sequences to BLAST, an algorithm for identifying homologous regions of sequence (Altschul et al., 1990), at the National Center for Biotechnology Information.

2.5. Northern blot analysis

Analysis of RNA was done on 1–1.2% formaldehyde agarose gels according to the procedure of Sambrook et al. (1982). RNA was blotted onto Nylon membranes (Tropilon, Bedford, MA) with a vacuum blotter (Bio-Rad, Richmond, CA). Blots were irradiated with 200 mJ/cm^2 in a UV crosslinker (Fisher Scientific, Houston, TX). An 842-bp oOR DNA probe was synthesized by PCR amplification of cloned oOR sequence with δOR395$^+$ and oOR1292$^-$. Unincorporated dNTPs were removed from the PCR product by column chromatography (ChromaSpin-100 column, Clontech, Palo Alto, CA), which was then radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) by nick translation (Promega Corp.).

Hybridization of radiolabeled probe to Northern blots was achieved at 55–60° C for 12–14 h (55° C for total RNA/60° C for poly(A)$^+$ RNA) while shaking in hybridization solution (50 ng labeled probe/ml, 30% formamide, 7% SDS, 120 mM NaH_2PO_4, 250 mM NaCl). Excess probe was removed from membranes by sequential 15-min washes in solutions of 2 × SSC/0.5% SDS, 0.5 × SSC/0.5% SDS, and twice in 0.1 × SSC/0.5% SDS. A final wash was performed at 55–60° C for 10 min in a 0.1 × SSC/0.5% SDS solution.

2.6. Southern blot analysis

Following electrophoresis, PCR products were vacuum-blotted and immobilized on Nylon membranes. Probe synthesis was performed as described above. The FACS lymphocyte RT-PCR products were hybridized with radiolabeled δOR395$^+$/oOR925$^-$ PCR product amplified from cloned oOR sequence. The *Bsu*36I-digested RT-PCR products were hybridized with radiolabeled oOR114$^+$/oOR925$^-$ PCR product amplified from cloned oOR sequence. Probe hybridization and membrane washes were performed as described above.

2.7. Antisense oligonucleotide experiments

Antisense oligonucleotide was prepared against the oOR 5′ untranslated region (i.e. oOR153$^-$, 5′-AGCCACTCAGTACAGTTC-3′), and a scrambled sequence of oOR153$^-$ (5′-ATCCCTTAATCGCGCAAG-3′) was generated to control for non-specific inhibition. These oligonucleotides were synthesized by LSUMC Core Laboratories.

Proliferation assays were performed, as follows: 5 × 10^5 splenic lymphocytes in 100 μl complete medium (RPMI 1640 containing 10% fetal bovine serum and 2.5% Hybri-Max (Sigma Chemical Company, St. Louis, MO) antibiotic/antimycotic solution) were placed in

96-well microtiter plates (Costar, Cambridge, MA). 100 μl complete medium containing lipopolysaccharide (LPS, 1.0 μg/well) was added to each well, along with 1.0 μg of antisense (oOR153$^-$) or scrambled oligonucleotide. Cells were cultured at 37° C/5% CO_2 for 48 h. [^{3}H]Thymidine (500 nCi) in 10 μl of Hank's balanced salt solution was added to each well, and the cells were cultured 16–24 h. Cells were harvested on glass fiber filter strips using a multi-well harvester (Cambridge Technologies, Watertown, MA). Filters were placed in scintillation vials containing 6.0 ml of Cytoscint liquid scintillation cocktail (ICN, Irvine, CA) and allowed to equilibrate for 2–4 h. The incorporation of [^{3}H]thymidine was determined by liquid scintillation counting using a Beckman LS9800. The mitogenic response of each treatment group was assayed in quadruplicate. Incorporation of [^{3}H]thymidine by cells cultured in the absence of LPS was less than 20% of that obtained in maximally stimulated cultures.

Antibody production from lymphocytes was determined, as follows: 2×10^6 splenic lymphocytes in 1.0 ml of complete medium were added to 24-well culture plates (Costar). Antisense (oOR153$^-$) or scrambled oligonucleotide was added to each well (5.0 or 2.5 μg) in 5 μl of phosphate-buffered saline, along with 2.0 μg of LPS. After 5 days incubation at 37° C/5% CO_2, supernates were harvested and assayed for polyclonal IgG or IgM by ELISA as previously described (Carr et al., 1990b).

3. Results

3.1. Screening lymphocytes for expression of OR mRNA

Splenic lymphocytes were screened for the expression of OR-encoding mRNA transcripts by RT-PCR. Initially, oligonucleotide primers δOR395$^+$ and δOR695$^-$ were made against the coding sequence of TM III and TM V of the δOR clone, DOR-1 (Evans et al., 1992). These primer sequences were chosen for screening lymphocytes because they are highly conserved among ORs. δOR395$^+$ is 83% and 70% homologous to the TM III-encoding sequence of rat κ- and μ- ORs, while δOR695$^-$ is 84% and 80% homologous, respectively, to the TM V coding sequence of these ORs.

RT-PCR amplification of lymphocyte RNA using the primers δOR395$^+$ and δOR695$^-$ yielded two major populations of PCR products. One PCR product corresponded in size to the predicted 301-bp DOR-1 product, while the other PCR product was approximately 80 bp larger. Upon Southern blotting, however, these lymphocyte-derived PCR products failed to anneal with a DOR-1-specific oligonucleotide probe (not shown). The PCR products were cloned and sequenced, and the 301-bp PCR product was found to be identical in sequence to bases 532 to 832 of an oOR clone isolated from a mouse brain cDNA library (Genbank accession number U04952). The 382-bp PCR product was also amplified from oOR mRNA, but it contained an additional 81 bases of sequence which lie between bases 748 and 749 of the oOR cDNA sequence (Fig. 1). These 81 bases formed an intron motif with 5′ splice donor (AAG$^\downarrow$GTCAGT) and 3′ splice acceptor (CTCTCCTGCAG$^\downarrow$) sequences.

```
          (δOR395+)→
532   GcTgtGCtCTcCctTgactactacaacatgtttaccagcactttcact
580 ttgactgccatgagtgtagaccgttatgtagctatctgccaccctatccg
630 tgcccttgatgttcggacatccagtaaagcccaggccgttaatgtggcca
680 tatgggccctggcttcggtggttggtgttcctgttgccatcatgggctca
730 gcacaagtggaggatgaag|gtcagtgggc agtcctcctc
          cctgaccaat cagttcccca tggttcttgc cggcccctct
          gacctcattt ctctcctgca g|agatcgagtgcctggtggagatccccgcccc
                                          ←(δOR695-)
780 tcaggactattggggccctgtatttgccatctgcGtGttcctctttGccttcG
```

Fig. 1. Nucleotide sequence of lymphocyte-derived oOR RT-PCR product obtained with δOR primers. The sequence of δOR395$^+$ and the complement of δOR695$^-$ are shown in boldface type. δOR primer bases that were not homologous with oOR are shown in uppercase letters. Bases are numbered according to their position in a mouse oOR cDNA clone. The 81-base intron which was present in the 382-bp oOR PCR product is shown in italics.

Using the same primers, δOR395$^+$ and δOR695$^-$, expression of oOR transcripts in brain and lymphocyte RNA was compared by RT-PCR (Fig. 2). Upon gel electrophoresis, the brain-derived PCR products (lane

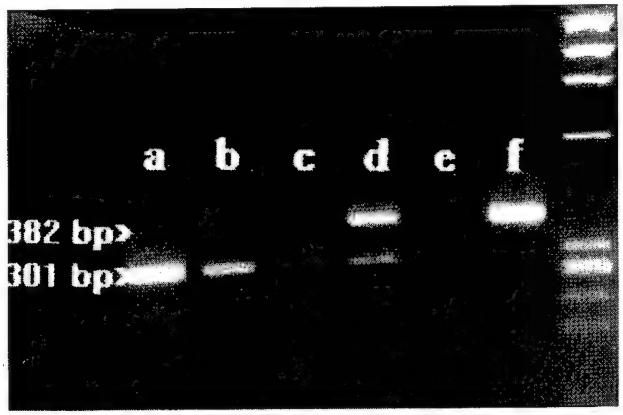

Fig. 2. Detection of oOR expression in brain and lymphocytes by RT-PCR analysis. RT-PCR was performed on RNA samples isolated from brain (lane a), whole spleen (lane b), unstimulated lymphocytes (lane c), and ConA-stimulated lymphocytes (lane d). No template (lane e) and cloned oOR sequence (lane f) served as negative and positive controls, respectively, of PCR amplification. ϕX174/*Hae*III markers are shown on the far right.

a) migrate as a single band of 301 bp while lymphocyte-derived PCR products (lanes b–d) separate into two distinct bands of 382 bp and 301 bp. Although the RT-PCR was not quantitative, the yield of 382-bp PCR product from ConA-stimulated lymphocyte RNA was markedly higher than that obtained from unstimulated lymphocyte RNA.

3.2. DNA sequencing of lymphocyte-derived oOR cDNAs

Having identified oOR mRNA in lymphocytes, oligonucleotide primers were prepared (i.e. oOR52$^+$, oOR114$^+$, oOR1292$^-$, and oOR1333$^-$) that flanked the open reading frame of the brain-derived oOR cDNA (Genbank accession number U04952). With these outer primers, nested PCR was used to amplify the remainder of the oOR coding sequence from lymphocyte cDNA. A RT-PCR product was obtained from lymphocyte RNA with the primers δOR395$^+$ and oOR1292$^-$. The sequence of this 842-bp PCR product also contained the previously identified 81-base intron, but was otherwise identical to the oOR cDNA clone.

```
                    (oOR114+)→
101                 gaggttgtgtgtgctgttggaggaa.....................

160 .gtgacagcatggagtccctctttcctgccccattctgggaggtcttgtatggcagccac
                M  E  S  L  F  P  A  P  F  W  E  V  L  Y  G  S  H
220 tttcaagggaacctgtctctcctaaatgagaccgtaccccatcacctgctcctcaatgct
     F  Q  G  N  L  S  L  L  N  E  T  V  P  H  H  L  L  L  N  A
280 agccacagtgccttcctgccccttggactcaaggtcaccatcgtggggctctacttggct
     S  H  S  A  F  L  P  L  G  L  K  V  T  I  V  G  L  Y  L  A
340 gtgtgcatcgggggctcctggggaactgcctcgtcat|gtatgtcatcctcag| gcacacc
     V  C  I  G  G  L  L  G  N  C  L  V  M  Y  V  I  L  R  H  T
400 aagatgaagactgctaccaacatttacatatttaatctggcactggctgataccctggtc
     K  M  K  T  A  T  N  I  Y  I  F  N  L  A II L  A  D  T  L  V
460 ttgctgacactgcccttccagggcacagacatccttctgggcttctggccatttgggaat
     L  L  T  L  P  F  Q  G  T  D  I  L  L  G  F  W  P  F  G  N
520 gcactgtgcaagacggtcattgctatcgactactacaacatgtttaccagcactttcact
     A  L  C  K  T  V  I  A  I  D  Y  Y  N  M III F  T  S  T  F  T
580 ttgactgccatgagtgtagaccgttatgtagctatctgccaccctatccgtgcccttgat
     L  T  A  M  S  V  D  R  Y  V  A  I  C  H  P  I  R  A  L  D
640 gttcggacatccagtaaagcccaggccgttaatgtggccatatgggccctggcttcggtg
     V  R  T  S  S  K  A  Q  A  V  N  V  A  I  W  A  L  A IV S  V
700 gttggtgttcctgttgccatcatgggctcagcacaagtggaggatgaag'agatcgagtgc
     V  G  V  P  V  A  I  M  G  S  A  Q  V  E  D  E  E  I  E  C
760 ctggtggagatccccgcccctcaggactattggggccctgtatttgccatctgcatcttc
     L  V  E  I  P  A  P  Q  D  Y  W  G  P  V  F  A  I  C  I  F
820 ctttttttccttcatcatcccggttctgatcatctctgtctgctacagcctcatgattcga
     L  F  S  F  I  I  P  V V L  I  I  S  V  C  Y  S  L  M  I  R
880 cgacttcgtggtgtccggctgctttcaggctcccgagagaaggaccggaacctgcgacgc
     R  L  R  G  V  R  L  L  S  G  S  R  E  K  D  R  N  L  R  R
940 atcacacggctggtactggtagttgtggctgtgtttgtgggctgctggacacctgtgcag
     I  T  R  L  V  L  V  V  V  A  V  F  V  G VI C  W  T  P  V  Q
1000 gtctttgtcctggttcaaggactgggtgttcagccaggtagtgagactgcagtagccatt
     V  F  V  L  V  Q  G  L  G  V  Q  P  G  S  E  T  A  V  A  I
1060 ctgcgcttctgcacagccctgggctatgtcaacagttgtctcaatcccattctctatgct
     L  R  F  C  T  A  L  G  Y  V  N  S VII C  L  N  P  I  L  Y  A
1120 ttcttggatgagaacttcaagcctgctttagaaagttctgctgtgcttctgccctgcac
     F  L  D  E  N  F  K  A  C  F  R  K  F  C  C  A  S  A  L  H
1180 cgggagatgcaggtttctgatcgtgtgcgcagcattgccaaggatgtaggccttggttgc
     R  E  M  Q  V  S  D  R  V  R  S  I  A  K  D  V  G  L  G  C
1240 .....................catgactaggcgtggacctgcccat
     K  T  S  E  T  V  P  R  P  A  COO-          ←(oOR1292-)
```

Fig. 3. Nucleotide sequence of pLoOR$^+$ PCR product insert. The sequence of oOR114$^+$ and the complement of oOR1292$^-$ are shown in boldface type. The predicted amino acid translation of oOR mRNA is shown below the nucleotide sequence. The underlined segments of amino acid sequence are the putative transmembrane spanning regions. Bases 378–392 are shown in a different font to denote their absence from the cloned insert in pLoOR$^-$. (▼) Site from which the 81 base intron is spliced. (···) Bases that are present in the mouse oOR cDNA clone, but whose presence we did not establish by sequencing.

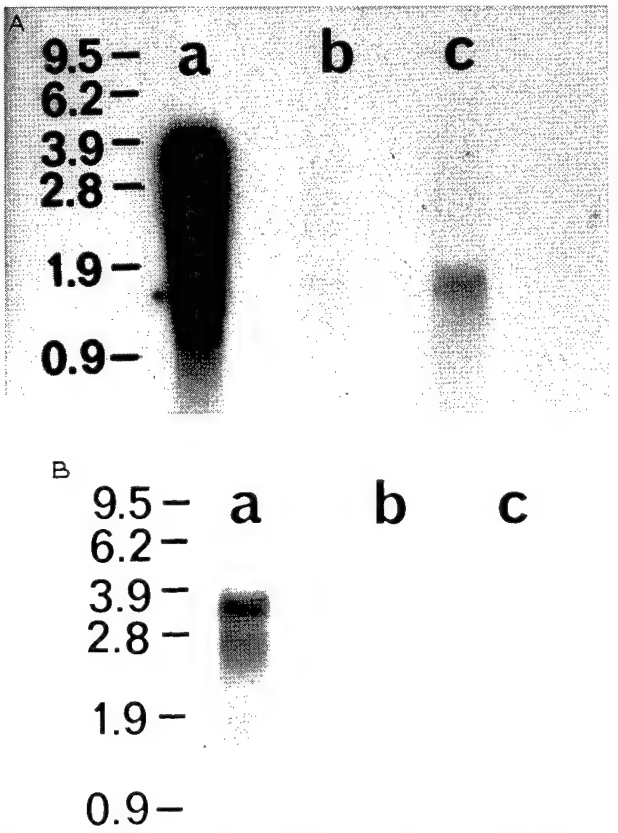

Fig. 4. Detection of oOR expression in brain and lymphocytes by Northern blot analysis. (**A**) 10.0 μg poly(A)$^+$ RNA from brain (lane a), unstimulated lymphocytes (lane b), and ConA-stimulated lymphocytes (lane c) hybridized with oOR probe. The position of RNA markers in the original gel is shown on the far left (size given in kilobases). (**B**) Shorter exposure of autoradiograph shown in (**A**).

A second PCR product obtained from lymphocyte cDNA using the primers oOR114$^+$ and oOR1292$^-$ included the entire coding sequence of the oOR transcript. Two copies of this lymphocyte-derived oOR114$^+$/oOR1292$^-$ PCR product were cloned into plasmids and sequenced. These plasmids are referred to hereafter as pLoOR$^-$ and pLoOR$^+$. The sequence of pLoOR$^+$ (Fig. 3) was identical to the mouse brain oOR cDNA (Genbank accession number U04952). The sequence of pLoOR$^-$, however, differed in that 15 bases of the oOR sequence were absent (bases 378–392). This region apparently formed a small intron motif that had been spliced out of the original oOR mRNA template from which pLoOR$^-$ was derived.

3.3. Northern blot analysis

Expression of oOR mRNA in splenic lymphocytes and brain was compared by Northern blot analysis. Two species of RNA transcripts, 3.0 and 1.5 kb, were readily detected in total brain RNA (data not shown). Despite the intense signal from the 3.0-kb transcripts

found in brain RNA, the 1.5-kb band correlates more closely in size to the 1338-base oOR cDNA clone isolated from mouse brain (Genbank accession number U04952), and the 1.3–1.8-kb cDNA clones isolated from rat brain (Bunzow and Grandy, 1994; Chen et al., 1994; Fukuda et al., 1994). Of the spleen-derived total RNA samples, ConA-stimulated but not unstimulated lymphocytes contained a detectable 1.5-kb oOR transcript. Hybridization of a G3PDH (i.e. housekeeping gene) probe to total RNA showed that less G3PDH mRNA was present in unstimulated lymphocytes than in brain and ConA-stimulated lymphocytes (data not shown), which presumably reflects the fact that lymphocytes are a relatively quiescent cell population in vivo, and are less transcriptionally active than brain and mitogen-stimulated lymphocytes.

Northern blot analysis of poly(A)$^+$ RNA (Fig. 4A) showed that 1.5-kb oOR mRNA was detectable in both unstimulated (lane b) and ConA-stimulated (lane c) splenic lymphocytes. A lighter exposure of the same Northern blot (Fig. 4B) shows that brain poly(A)$^+$ RNA contained three predominant mRNA species (1.5, 3.0, and 4.0 kb) that hybridized with oOR probe. Based on phosphorimager densitometric analysis, the unstimulated and ConA-stimulated splenic lymphocyte poly(A)$^+$ RNA samples contained 4.5% and 22.5% as much 1.5 kb oOR mRNA as was found in the brain.

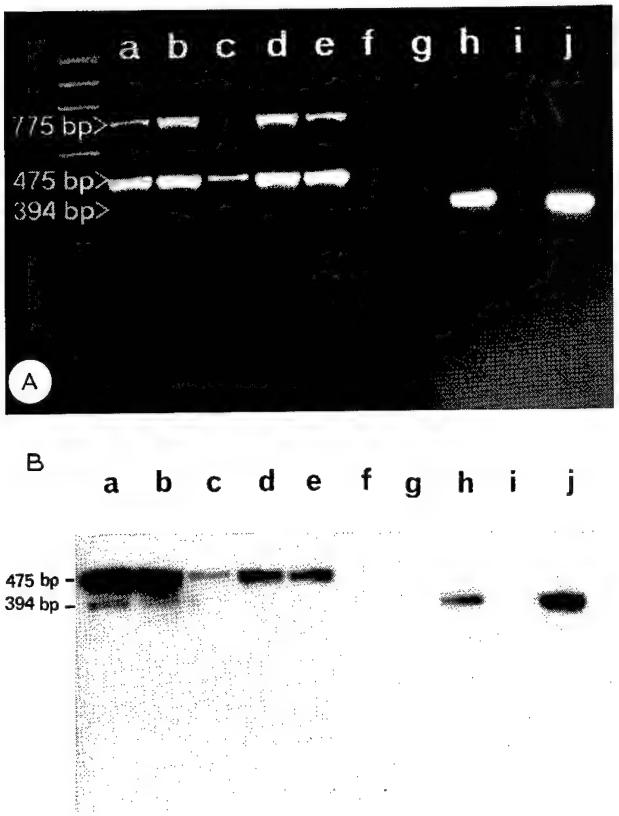

Fig. 5. Detection of oOR expression in CD4$^+$, CD8$^+$, and CD4$^-$CD8$^-$ lymphocytes by RT-PCR analysis. **(A)** Agarose gel analysis of δOR395$^+$/oOR925$^-$ RT-PCR products. RNA samples were isolated from cell-sorted CD4$^+$, CD8$^+$, CD4$^-$CD8$^-$ lymphocytes (lanes a, b, c); total cells prior to sorting (lane d), Lympholyte M®-purified lymphocytes (lane e), EL4 cells (lane f), L929 cells (lane g), and brain (lane h). No template (lane i) and cloned oOR sequence (lane j) served as negative and positive controls, respectively, of PCR amplification. φX174/*Hae*III markers are shown on the far left. **(B)** Southern blot of the above gel hybridized with oOR probe.

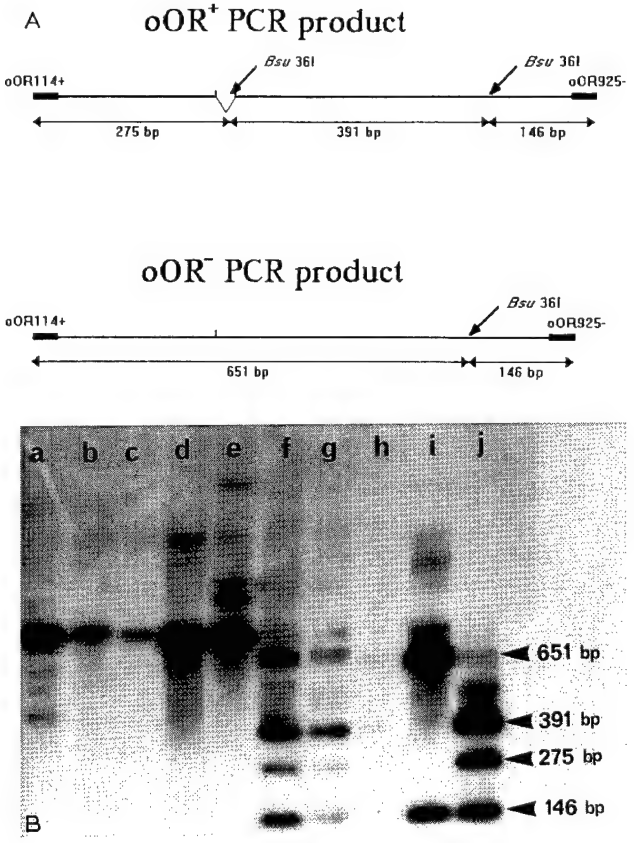

Fig. 6. *Bsu*36I restriction analysis of oOR RT-PCR products. **(A)** *Bsu*36I restriction map of oOR114$^+$/oOR925$^-$ RT-PCR products amplified from oOR$^+$ and oOR$^-$ mRNA. **(B)** The Southern blot shown contains untreated oOR114$^+$/oOR925$^-$ PCR products (lanes a–e), and aliquots of the same samples following digestion with *Bsu*36I (lanes f–j). These RT-PCR products were amplified from brain poly(A)$^+$ RNA (lanes a and f), brain total RNA (lanes b and g), lymphocyte total RNA (lanes c and h). The standards for the *Bsu*36I restriction digests were PCR products amplified from pLoOR$^-$ (lanes d and i), and pLoOR$^+$ (lanes e and j). A control PCR tube without template did not yield any products (not shown).

3.4. Expression of oOR mRNA in lymphocyte subpopulations

In order to assess oOR mRNA expression in lymphocyte subpopulations, RT-PCR was performed using RNA from CD4$^+$, CD8$^+$, and CD4$^-$CD8$^-$ cell-sorted lymphocytes (Fig. 5A). A Southern blot of the resulting PCR products is shown in Fig. 5B. Both brain RNA (lane h) and pLoOR$^-$ (lane j) yielded a PCR product

of the predicted 394-bp size. The majority of oOR PCR products obtained from the lymphocyte-derived RNA samples (lanes a–e), however, were 475 bp, indicating that the 81-base intron motif was present. A non-oOR PCR product (i.e. one that did not hybridize with the oOR probe) of approximately 775 bp was also amplified from $CD4^+$, $CD8^+$, and total lymphocyte RNA (lanes a, b, and d–e), but not from $CD4^-CD8^-$ lymphocytes nor any of the other RNA samples (Fig. 5A). Based on RT-PCR analysis (lanes a–c), oOR mRNA appears to be expressed in all three lymphocyte subpopulations.

3.5. Restriction analysis of oOR RT-PCR products

The absence of a 15-base intron motif (bases 378–392) in $pLoOR^-$ suggested that alternative splicing of oOR mRNA occurs in lymphocytes. Because a *Bsu*36I restriction site (CCTNAGG, bases 387–393) occurs in the intron, RT-PCR products amplified from oOR mRNA lacking bases 378–392 did not contain the restriction site. Therefore, *Bsu*36I restriction analysis provided a method by which RT-PCR products amplified from oOR^+ mRNA (bases 378–392 present) could be differentiated from RT-PCR products amplified from oOR^- mRNA (bases 378–392 absent). Restriction maps of oOR^+ and oOR^- PCR products obtained in these experiments are shown in Fig. 6A.

In Fig. 6B, a radiolabeled oOR probe was hybridized to untreated $oOR114^+/oOR925^-$ PCR prod-

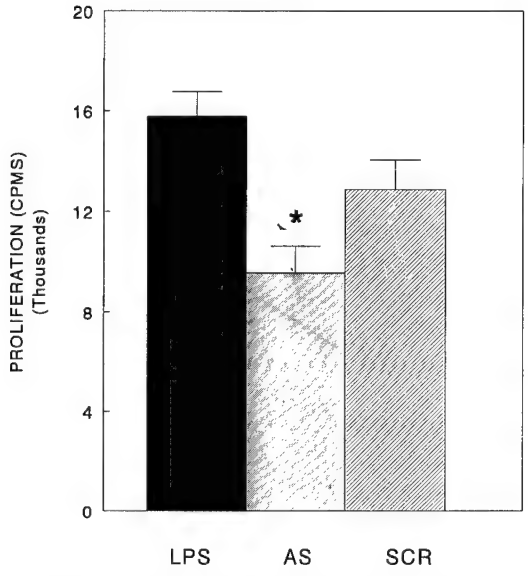

Fig. 7. Orphan OR antisense oligonucleotide blocks LPS-induced lymphocyte proliferation. Splenic lymphocytes (5×10^5 cells) were stimulated with LPS in the presence or absence of antisense OR oligo (AS, 1.0 μg) or scrambled oligo (SCR, 1.0 μg). Cells were then assessed for proliferation by [^{3}H]thymidine uptake 72 h following initiation of culture. Bars represent S.E.M. This figure represents the summary of three independent experiments with each condition measured in quadruplicate/experiment. * $P < 0.05$, $F(2,8) = 8.4459$ comparing the oligo-treated cultures to LPS only culture as determined by ANOVA and Scheffé multiple comparison test.

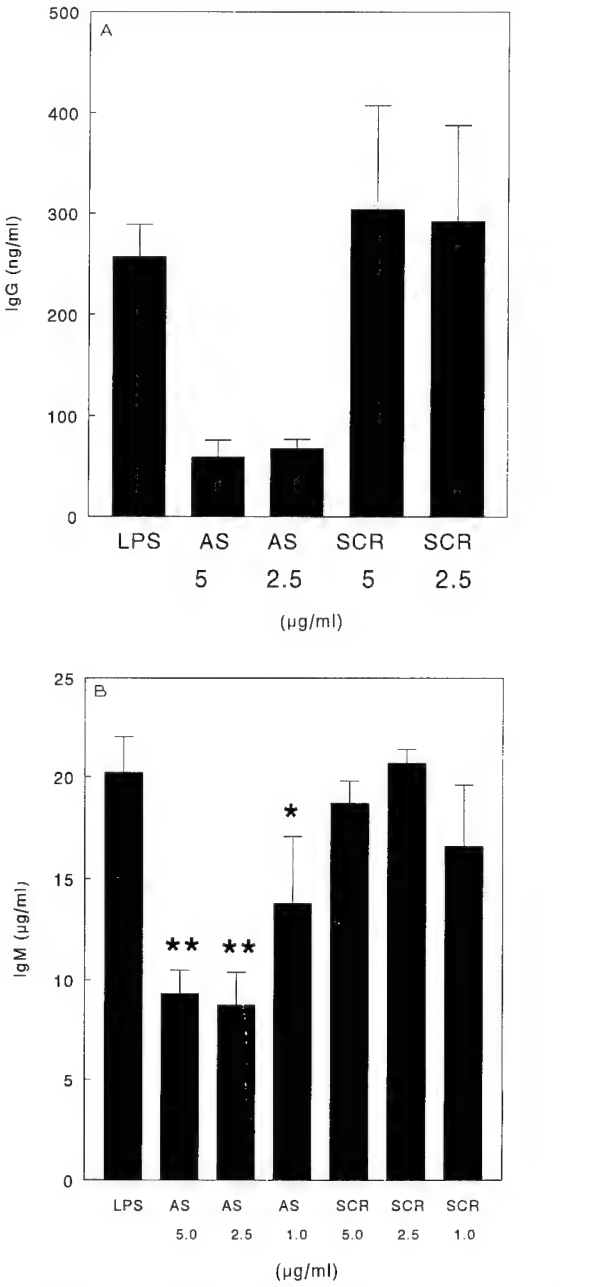

Fig. 8. Orphan OR antisense oligonucleotide blocks LPS-induced polyclonal antibody production. Splenic lymphocytes (2×10^6 cells) were stimulated with LPS in the presence or absence of the indicated concentration of antisense (AS) or scrambled (SCR) oligos. Supernates were collected 5 days following the initiation of culture and assayed for polyclonal IgG (**A**) or polyclonal IgM (**B**). Bars represent S.E.M. The figures are a summary of three independent experiments with each condition measured in triplicate/experiment. * $P < 0.05$, ** $P < 0.01$, $F(6,20) = 6.1263$ as determined by ANOVA and Tukey's post t-test comparing the oligo-treated cultures to the LPS-only culture.

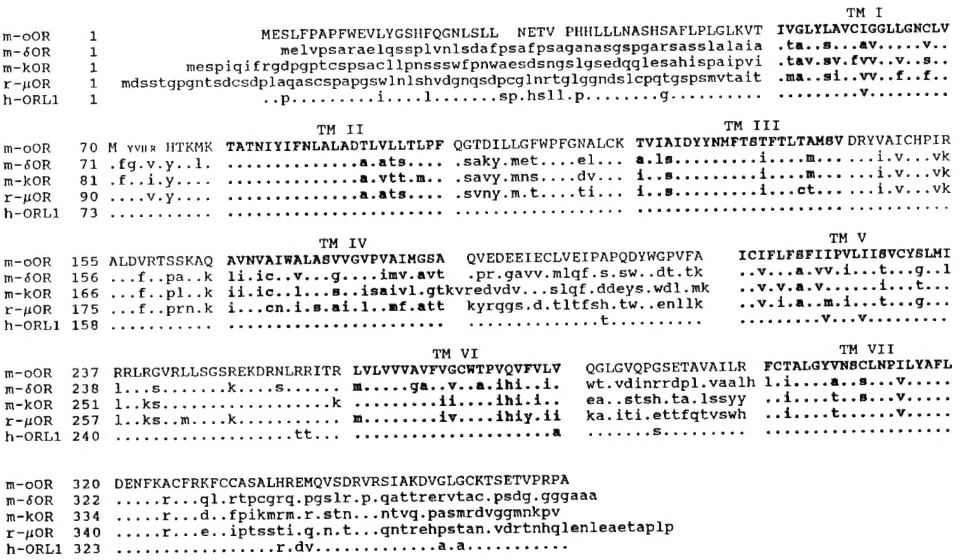

Fig. 9. Alignment of the mouse oOR sequence with other opioid receptors. The other sequences are mouse δ- and κ- ORs (Yasuda et al., 1993), rat μOR (Wang et al., 1993) and human oOR (Mollereau et al., 1994). (·) Residues that are identical to the corresponding oOR amino acid. TM spanning regions are shown in boldface type.

ucts (lanes a–e) and *Bsu*36I-digested oOR114$^+$/oOR925$^-$ PCR products (lanes f–j). The untreated PCR products (lanes a–e) all migrated at a rate corresponding to the predicted size of 812 bp. All oOR114$^+$/oOR925$^-$ PCR products treated with *Bsu*36I were cleaved into 146-bp (lanes f–j) and either 651-bp or 666-bp fragments because of a restriction site which occurs at bases 778–784. The 651-bp PCR product fragments derived from pLoOR$^-$ (lane i) and oOR$^-$ mRNA (lanes f–h) lacked the intron and were not further cleaved by *Bsu*36I. However, the 666-bp PCR product fragments amplified from pLoOR$^+$ (lane j) and oOR$^+$ mRNA (lanes f–h) contained the intron, and were thus cut into 275-bp and 391-bp fragments by *Bsu*36I.

The amplification of *Bsu*36I-insensitive and *Bsu*36I-sensitive RT-PCR products (lanes f–h) indicates that both oOR$^-$ and oOR$^+$ species of mRNA are present in brain and lymphocytes. Using phosphorimager densitometric analysis, we estimated the relative amounts of oOR$^+$ and oOR$^-$ RT-PCR products amplified from brain and lymphocyte RNA. The ratio of oOR$^+$:oOR$^-$ products that was measured in each RNA sample is listed, as follows: brain poly(A)$^+$ = 2.2, brain total = 2.9, and lymphocyte total = 1.2. Because oOR$^+$ and oOR$^-$ mRNA are nearly identical, amplification of these sequences should proceed at equal rates. Therefore, the relative yield of oOR$^+$ and oOR$^-$ RT-PCR products reflects the underlying ratio of oOR$^+$ to oOR$^-$ mRNA in the cells. Based on this assumption, the fraction of oOR mRNA which lacked bases 378–392 in each of the RNA samples is listed, as follows: brain poly(A)$^+$ = 0.32, brain total = 0.26, and lymphocyte total = 0.45.

3.6. Antisense oligonucleotide defines a functional role for lymphocyte oOR

Lymphocytes have been reported to secrete endorphins following LPS stimulation (Harbour-McMenamin et al., 1985). To determine the potential relationship between lymphocyte-derived endorphins and orphan opioid receptors on immune function, antisense oligonucleotides were used to block oOR expression. Lymphocytes were stimulated with LPS in the presence of either oOR153$^-$ (oOR-antisense oligonucleotide) or a scrambled control sequence, and their effects on proliferation and antibody production were compared. Orphan OR153$^-$ significantly reduced the LPS-stimulated proliferation of splenic lymphocytes by 49.9 ± 1.7% (Fig. 7). By comparison, the scrambled oligonucleotide inhibited LPS-induced proliferation as well (22 ± 8.0%) (Fig. 7). Similarly, oOR153$^-$ significantly inhibited (50–75%) polyclonal IgG and IgM production from LPS-stimulated splenic lymphocytes, but the scrambled oligonucleotide had no effect (Fig. 8).

3.7. Nucleotide sequence

This sequence has been deposited in the Genbank database under accession number U14165.

4. Discussion

There is evidence that δORs are present on lymphocytes (Carr, 1991), but this has not been confirmed at the level of gene expression. To address this point, we screened lymphocyte RNA for the presence of

δOR-encoding RNA transcripts by RT-PCR. δOR mRNA was not detected, but rather the selected primer pair detected expression of oOR mRNA. Overall, oOR shares 61% amino acid identity with δOR and 58% with μ- and κ- ORs, but sequence conservation amongst the ORs is particularly high within the TM spanning regions (Fig. 9). When the comparison of sequence homology is extended to analogous amino acids, oOR shares 73%, 74%, and 76% similarity with μ-, κ-, and δ-ORs, respectively.

Functional expression of the human oOR cDNA clone, hORL1, in COS cells showed that oOR did not bind endogenous opioid ligands. However, the universal opiate agonist etorphine did bind and cause suppression of adenylate cyclase activity in COS cells, and these effects were sensitive to diprenorphine, an opioid antagonist (Mollereau et al., 1994). In situ hybridization indicates that differential expression of oOR occurs in rat brainstem (Fukuda et al., 1994), suggesting a role for oOR in modulation of neurotransmission in the neuroendocrine system.

Exogenous (Bussiere et al., 1993) and endogenous (for review, Carr, 1991) opioids have been shown to modulate antibody production. LPS has been shown to induce leukocyte production of endogenous opioids and the endorphins (Harbour-McMenamin et al., 1985). In the present study, oOR-specific antisense oligonucleotides blocked antibody production from splenic lymphocytes, and had a modest effect on proliferation. The results suggest that oORs play a role in autocrine regulation of lymphocyte function. Therefore, lymphocyte oORs are potentially significant sites of immunoregulation.

Post-transcriptional processing of oOR RNA transcripts appears to be different in lymphocytes than in brain. RT-PCR of brain RNA with $\delta OR395^+$ and $oOR925^-$ yields a 394-bp PCR product, while lymphocyte RNA yields 475-bp and 394-bp oOR-derived PCR products. The difference in size presumably reflects the presence (475 bp) or absence (394 bp) of an 81-base intron in the original oOR RNA template. Therefore, while brain-derived oOR transcripts are fully processed, an 81-base intron is present in a large fraction of lymphocyte-derived oOR transcripts. Given that the extra 81 bases would not disrupt the open reading frame, this RNA species could have encoded an oOR subclass with an additional 27 amino acids in the second extracellular loop. Translation of the 81-base sequence, however, revealed that a UGA stop codon was present (verified in three independent clones), making it unlikely that this oOR RNA species encodes a functional oOR. The existence of this immature oOR RNA species suggests that lymphocyte expression of oOR may be regulated post-transcriptionally at the level of mRNA splicing.

Sequence analysis of two $oOR114^+/oOR1292^-$ PCR products revealed that a 15-base intron motif (bases 378–392) which is present in all identified oOR cDNA clones (Bunzow and Grandy, 1994; Chen et al., 1994; Fukuda et al., 1994; Mollereau et al., 1994) was spliced from $pLoOR^-$. *Bsu*36I restriction analysis of $oOR114^+/oOR925^-$ RT-PCR products confirmed that alternative splicing of oOR mRNA occurs in brain and lymphocytes. Assuming that RT-PCR did not preferentially amplify one oOR splice variant, phosphorimager densitometric analysis indicated that bases 378–392 are removed from 25–30% of oOR mRNA in brain, and approximately 45% of oOR mRNA in lymphocytes.

Pharmacological characterization indicates that there is more than one type of δ-, κ-, and μ-OR. Likewise, alternative splicing of bases 378–392 of oOR mRNA which encode Y^{71}-R^{75} suggests that at least two functionally distinct oOR subtypes exist. For example, removal of Y^{71}-R^{75} from the first intracellular loop may sterically alter oOR, thereby changing the ligand affinity of this receptor subtype. Likewise, removal of a potential tyrosine kinase substrate (i.e. YXX[L/I] are phosphorylation sites in antigen recognition activation motifs found in a variety of lymphocyte-derived receptors, e.g. the CD3 ε and γ chains) could produce an oOR subtype that is differentially regulated.

Northern blot analysis confirmed that a 1.5-kb oOR mRNA transcript is expressed in both stimulated and unstimulated murine splenic lymphocytes. Because $poly(A)^+$ RNA was isolated from unfractionated spleen cells, oOR mRNA could have potentially been derived from non-lymphoid cells in the spleen. However, the observed induction of oOR mRNA expression by ConA suggests that lymphocytes are the major source of oOR mRNA in the $poly(A)^+$ RNA blot. Furthermore, this hypothesis is supported by RT-PCR analysis of FACS-purified spleen cells which detected oOR mRNA in cells bearing $CD4^+$ and $CD8^+$ lymphocyte differentiation markers.

In conclusion, a gene encoding an opioid-like receptor is expressed in mouse lymphocytes. This finding lends support to pharmacological studies that have identified opioid binding sites on lymphocytes (Madden et al., 1987; Carr et al., 1989; Ovadia et al., 1989; Bidlack et al., 1992), as well as in vitro results which indicate that endogenous opioids modulate lymphocyte function (Johnson et al., 1982; Mathews et al., 1983; Van Epps and Saland, 1984; Heijnen et al., 1986; Carr and Klimpel, 1986; Mandler et al., 1986; Taub et al., 1991). Lymphocyte expression of neuropeptide receptors has been proposed as a mechanism by which neuroendocrine regulation of immune function occurs (Carr, 1991). Having identified and sequenced an opioid-like receptor mRNA in lymphocytes, further studies can begin to explore the function of oOR in neuroimmunomodulation.

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